# Exhibit B

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# Enhancement of Ocular Drug Penetration

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**ABSTRACT:** Although new drugs have recently been developed within the field of ophthalmology, the eye's various defense mechanisms make it difficult to achieve an effective concentration of these drugs within the eye. Drugs administered systemically have poor access to the inside of the eye because of the blood–aqueous and blood–retinal barriers. And although topical instillation of drugs is very popular in ophthalmology, topically applied drugs are rapidly eliminated from the precorneal area. In addition, the cornea, considered a major pathway for ocular penetration of topically applied drugs, is an effective barrier to drug penetration, since the corneal epithelium has annular tight junctions (zonula occludens), which completely surround and effectively seal the superficial epithelial cells. Various drug-delivery systems have been developed to increase the topical bioavailability of ophthalmic drugs by enhancement of the ocular drug penetration. The first approach is to modify the physicochemical property of drugs by chemical and pharmaceutical means. An optimum promoiety can be covalently bound to a drug molecule to obtain a prodrug that can chemically or enzymatically be converted to the active parent drug, either within the cornea or after the corneal penetration. Along these same lines, the transient formation of a lipophilic ion pair by ionic bonding is also useful for improving ocular drug penetration. The second approach is to modify the integrity of the corneal epithelium transiently by coadministration of an amphiphilic substance or by chelating agents that act as drug-penetration enhancers. The third approach modifies the integrity of the corneal epithelium transiently by physical techniques including iontophoresis and phonophoresis. This paper reviews the absorption behavior and ocular membranes penetration of topically applied drugs, and the various approaches for enhancement of ocular drug penetration in the eye.

**KEYWORDS:** Drug-delivery system, ocular penetration, enhancer, prodrug, iontophoresis, eye, promoter, ion pair, pharmacokinetics, barrier.

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## I. INTRODUCTION

Recently, a number of new drugs have been developed in the field of ophthalmology, including long acting β-blockers, α-1 agonists, prostaglandin derivatives, topical carbonate anhydrase inhibitors, and several peptide drugs. Most of these drugs were designed to act either within the eye or on the surface tissues only, and thus it is important to deliver a sufficient concentration of drug into the target site of the eye for effective medication. However, the eye is protected by a series of complex defense mechanisms. From a therapeutic standpoint, these defense mechanisms make it difficult to achieve an effective concentration of drug within the target area in the eye. Systemically administered drugs have poor access because of the blood–aqueous barrier, which prevents drugs from entering into the aqueous humor, and the blood–retinal barrier, which prevents drugs from entering into the extravascular space of the retina and into the vitreous body.[1,2] Subconjunctival injection and intravitreous injection of drugs are also applied clinically, but these methods are still characterized by poor bioavailability and are both painful and inconvenient.

Topical delivery of eye drops into the lower cul-de-sac is the most common method of drug treatment for ocular diseases and diagnoses because of its convenience and safety. However, the anterior segment of the eye also has various protective mechanisms for maintaining visual functions including cleaning of the inner and outer surfaces of the eye and elimination of foreign substances. Accordingly, after instillation

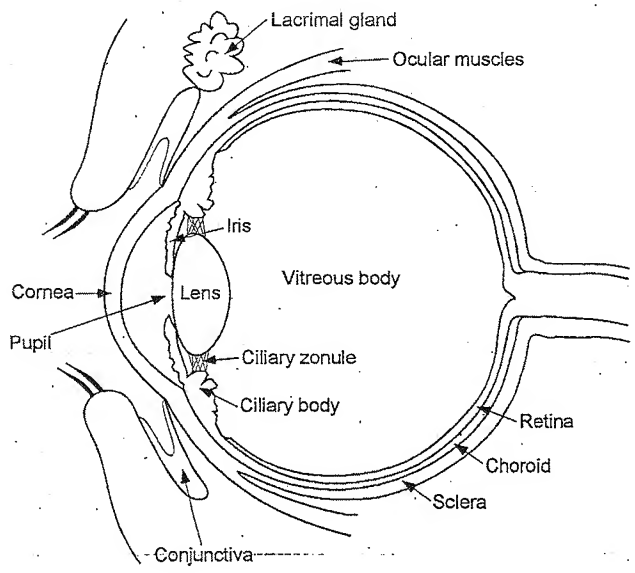



FIGURE 1. Schematic illustration of the eye.

of an ophthalmic drug, most of it is rapidly eliminated from the precorneal area due to drainage by the nasolacrimal duct and dilution by tear turnover; the removed drug is then readily absorbed into the systemic circulation.[3,4] Furthermore, the cornea, despite its protection by the tight barrier of the epithelium, is considered to be a major pathway for ocular penetration of topically applied drugs.[5,6,7] This obstructed pathway results in poor bioavailability and increases the severity of the systemic adverse effects of topically applied drugs.

Various drug delivery systems have been developed within the field of ophthalmology to improve the topical bioavailability of drugs.[3,8] One effective approach is to discourage drug drainage from the precorneal area by application of viscous solutions, suspensions, ointments, mucoadhesive gels, polymeric inserts, polymeric particles, and liposomes. Another promising approach is to enhance the penetration of drug through the tight barrier of the cornea by chemical, pharmaceutical, and physical approaches.

This paper reviews the absorption behavior and membrane penetration of drugs and the various approaches by which the penetration may be improved. The approaches primarily include the use of prodrugs, ion pairs, absorption enhancers, iontophoresis, and phonophoresis.

## II. ANATOMY AND PHYSIOLOGY OF THE EYE

Figure 1 shows the anatomy of that portion of the eye that is responsible for vision. The eyeball, an elongated sphere about 2.5 cm in diameter, has three layers: the sclera, choroid, and retina. At either end of its length, the choroid forms a ring-shaped ciliary body that controls the shape of the lens. The vitreous body fills the posterior cavity behind the lens. The iris regulates the size of the pupil. The cornea, the humors, and especially the lens bring light rays into focus on the retina. Recently, the concept of the ocular surface area was developed to describe the combined barrier mechanisms of the cornea, conjunctiva, and tear film. These mechanisms include a physical barrier resulting from the morphology of the membrane epithelium, a nonspecific antibiotic barrier caused by lysozyme and lactoferrin, and an immunological barrier in the conjunctiva and lacrymal glands. Thus the structural and functional characteristics of the eye can affect the intraocular absorption of topically applied drugs.

### A. Precorneal Area

The precorneal tear film covering the cornea and conjunctiva is the first structure encountered by topically applied drugs. The tear film consists of three main layers, as depicted in Figure 2. The outer layer is an oily and lipid layer secreted by the Meibomian glands and mainly prevents the evaporation of the tear fluid. The middle layer

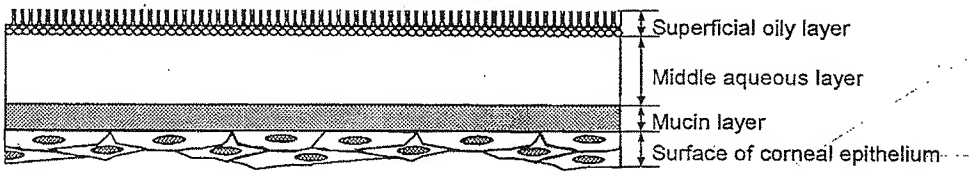



**FIGURE 2.** Schematic illustration of the precorneal tear film.

of the tear film is an aqueous layer containing salt solution and secreted by the main and accessory lacrymal glands. The inner layer is a mucous layer containing a family of glycoproteins and produced by the conjunctival goblet cells and the lacrymal gland. The mucin layer is important for wetting the corneal and conjunctival epithelium. The precorneal tear film formed by these layers is spread across the surface of the eye by blinking.

The normal volume of tear fluid contained by the ocular adnexa is 7–9 μL.[9,10] The maximum volume of fluid that can be contained in the cul-de-sac without overflow is approximately 30 μL. The normal commercial eye dropper delivers a drop volume of approximately 50 μL. The instilled solution is rapidly removed by spillage from the conjunctival sac or loss through the puncta to the lacrymal drainage system until the tear fluid returns to its normal volume. Normal tear flow varies widely in humans, with the average basal secretion being 1.2–1.5 μL/min. Ocular administration of irritating drugs or vehicles induces lacrimation as a reflex secretion that varies from 3 to 400 μL/min, according to the irritating power of the instilled vehicle.

The elimination of drug in the precorneal area is important for determining the ocular bioavailability and systemic side effect.[4,11] The loss of drug from the precorneal area is a net effect of the drainage, tear turnover, noncorneal absorption, and corneal absorption-rate processes. The drainage rate is much faster than the corneal absorption rate, with the result that most of the topically applied drug is eliminated from the precorneal area within 90 seconds. Chrai et al.[12] determined the influence of drop size on the elimination rate of instilled volume. The instilled volume was apparently reduced at the first order elimination rate constant according to the drop size. They found that the elimination rate constant of the instilled volume is directly proportional to the volume of lacrimal fluid in excess of the normal volume, and used this relationship to derive an equation for describing the drug concentration in precorneal area.

The tear flow associated with a loss of topically applied drug is influenced by other factors. The topically applied drug can be retained at the precorneal area by occluding the nasolacrimal duct, by placing the head in various positions, or by closing the eyelids.[13] In addition, ointment, benzalkonium chloride, cocaine, and dry eye

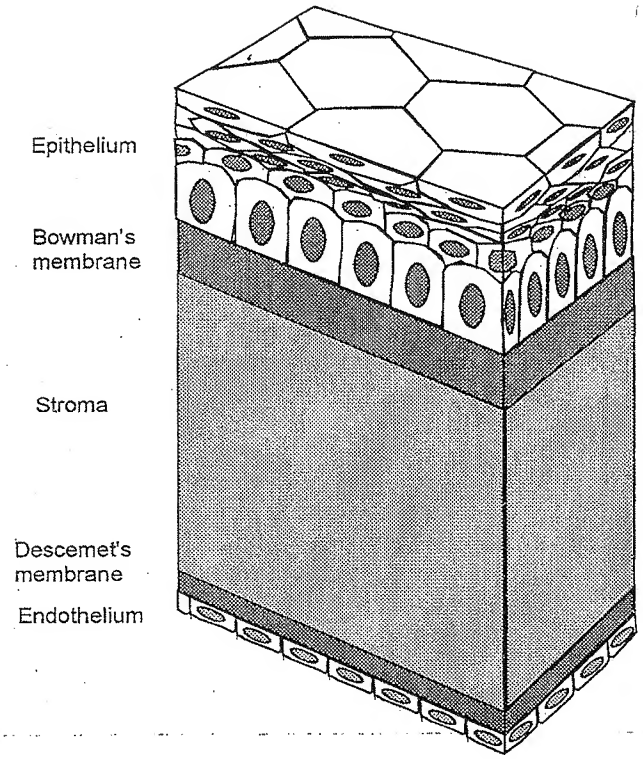



FIGURE 3. Schematic illustration of the cornea.

can diminish the break up time of tears,[14] while artificial tears increase it for a few minutes after instillation. Protein binding of drugs in the tear fluid is another factor affecting drug bioavailability.[15] The total protein content of human tear fluid ranges from 0.6% to 2% and is composed of albumin, globulin, and lysozyme.

## B. Ocular Membranes

### *1. Cornea*

The healthy cornea is an optically transparent tissue that acts as the principal refractive element of the eye. It is avascular, is nonpigmented except at the periphery (limbus), and is richly supplied with long ciliary sensory nerves, particularly pain receptors. The cornea responds to touch by rapid blinking, a sensitivity that both protects it and helps maintain its transparency. The corneal diameter is approximately 12 mm, with a radius of curvature of the anterior surface of approximately 8 mm and a mean thickness of approx-

imately 500 μm.[16] The cornea is composed of an epithelium, a Bowman's membrane, a stroma, a Descemet's membrane and an endothelium (Figure 3).

The corneal epithelium consists of 5–6 cell layers of nonkeratinized squamous cells. The epithelium consists of a basal layer of columnar cells, 2–3 layers of wing cells, and 1 or 2 outermost layers of squamous, polygonal-shaped superficial cells. The mean thickness is approximately 50 μm.[16] Cell divisions occur in the basal layer, and cellular differentiation occurs gradually as cells move towards the corneal surface. The outer layer of the surface cells possess microvilli on their anterior surface that presumably help to anchor the precorneal tear film. The cells produce an adhesion complex that maintains the strong binding of the cell layers and prevents physical disruption by the eyelids. The stratified corneal epithelium acts both as a protective barrier to prevent invasion of foreign substances and as a barrier to ion transport. The Bowman's membrane is a thin structure consisting of a fibrillar material that is probably mostly collagen.

The stroma, which represents 90% of the thickness of the cornea, is basically cellular, hydrophilic, and porous. It is composed mainly of collagen fibrils with a uniform diameter of 25–35 nm that run parallel to each other to form collagen bundles of varying widths and thicknesses. The stroma can be considered a comparatively open structure typically allowing diffusion of solutes of molecular weight below 500,000. The Descemet's membrane is the basement membrane of the endothelial cells. The corneal endothelium is a single layer of hexagonal cells covering the posterior surface of the cornea and facing the anterior chamber. The endothelial cell layer contains loose occludents and pinocytotic vesicles. The corneal endothelium is responsible for maintaining a normal corneal hydration by $Na^+$–$K^+$ ATPase, ionic conditions, and $Ca^{2+}$ homeostasis. Its role in overall barrier resistance is less significant.

The cornea is considered to be a major pathway for ocular penetration of topically applied drugs. Doane et al.[5] determined the concentration of steroid and pilocarpine in ocular tissues when drugs were separately applied on the cornea or on a noncorneal ocular area of rabbits using a plastic cylindrical well. The drug concentration in the iris–ciliary body after application on the cornea was higher than that after application on a noncorneal ocular area by a factor of 40 for steroid and 5 for pilocarpine. The cornea prevents drug penetration by means of a trilaminate structure consisting of a hydrophilic stromal layer sandwiched between a very lipophilic epithelial layer and a much less lipophilic endothelial layer. Drugs that are both lipid- and water-soluble pass through the cornea readily. Huang et al.[17] measured the permeability constant of various β-blockers across the intact cornea, the stroma alone, the epithelium-stroma, and the stroma-endothelium and determined the resistance to penetration for each corneal layer in rabbits. As shown in Figure 4, the epithelium was a rate-determining barrier for hydrophilic compounds. Lipophilic compounds penetrated the excised cornea more rapidly, although the stroma became a rate-determining barrier for the most lipophilic compounds.

Sasaki et al.[18] also analyzed the permeability of β-blockers through the cornea, the stromal and endothelial layer, and the conjunctiva in rabbits using Fick's equa-

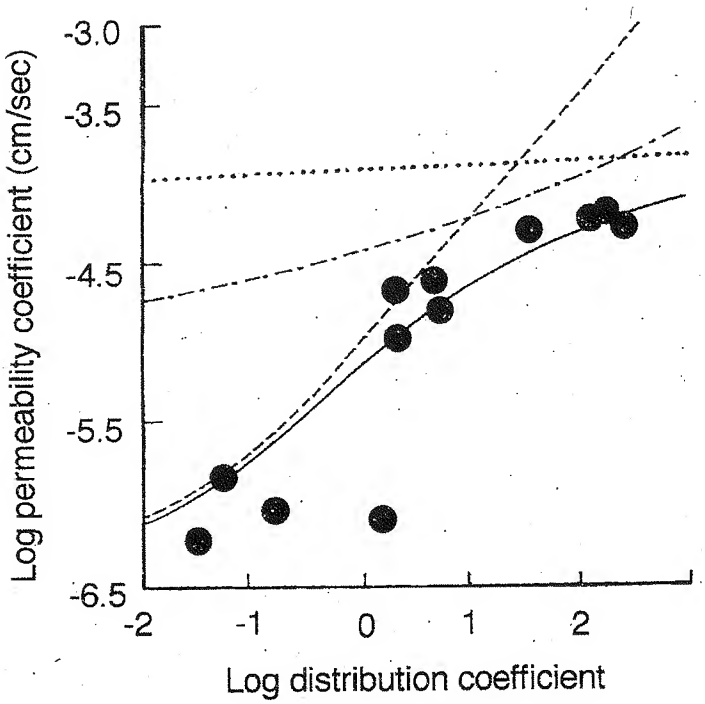



FIGURE 4. Permeability of the rabbit cornea to β-blockers. Relationship of the logarithms of the distribution coefficients (octanol-pH 7.65 buffer) and the logarithm of the permeability coefficients. Key: (●) intact corneal permeability data. Simulation curves: (- - - -) epithelium; (.....) stroma; (— — —) endothelium; (——) intact cornea. (From Huang et al.[17] Reproduced with permission of the American Pharmaceutical Association.)

tion. The drug penetration was characterized by the processes of diffusion and partition. The epithelial layer showed lower diffusion coefficients than the stromal and endothelial layers, in which the partition coefficients of β-blockers were close to unity. The lipophilic character of the corneal barrier contributed to a partition parameter rather than a diffusion parameter. These authors also reported that the conjunctival and scleral permeabilities were not susceptible to the lipophilicity of β-blockers.

## *2. Conjunctiva and Sclera*

The conjunctiva of the eyelids and globe is a thin, vascularized mucous membrane. The continuous surface area (18 $cm^2$) is about 17 times greater than the surface area of the cornea in humans.[19] The conjunctiva consists of a stratified squamous epithe-

lium, which is not keratinized, overlying a loose, highly vascular connective tissue. The cells of the superficial layer have numerous microvilli that are covered by a glycocalix and mucin. The superficial conjunctival epithelium has tight junctions that are the main barrier for drug penetration across this tissue. However, the intercellular spaces in the conjunctival epithelium are wider than those in the corneal epithelium. Therefore, the conjunctiva is more permeable than the cornea because of a richness of paracellular routes. Huang et al.[20] showed that the conjunctiva was much more permeable to hydrophilic macromolecules and $^{3}$H-mannitol than the cornea. Hamalainen et al.[21] estimated that the total paracellular space in the conjunctiva was 230 times wider (2 times larger pores and 16 times higher pore density) than that in the cornea. On the other hand, Hosoya et al.[22] demonstrated that the rabbit conjunctival epithelium had a high resistance of over 1,000 ohm/cm$^2$ and expressed forms of active transport such as $Na^+$-dependent glucose cotransport and $Na^+$-dependent L-arginine transport. After entering the conjunctival tissue, most drugs are rapidly removed by systemic uptake by the vessels embedded in that tissue.[6,7] This vascular clearance may be very important for *in vivo* absorption of drugs through the conjunctiva. Coadministration of vasoconstrictors decreases the systemic absorption of topically applied drug.[23] Conjunctival and scleral permeability contributes to noncorneal ocular absorption, which appears to be the route favored by the hydrophilic and by the large molecules that are poorly absorbed through the cornea.[5,24] The noncorneal route may be most important for drug absorption from adhesive gels and polymer inserts.

The outermost tunic of the eye is the sclera, which protects both the cornea and the sensitive inner parts of the eye. The sclera is a relatively resistant structure about 0.5–1.0 mm thick (being thicker at the posterior pole), and consisting mainly of collagen bundles and some elastic fibers. There are very few blood vessels in the sclera. The extraocular muscles insert their tendons into both the sclera and the Tenon's capsule, a loose collagenous structure covering the sclera. Scleral resistance to drug penetration is lower than that of the multilayered cornea. Scleral permeability to polyethylene glycol oligomers has been shown to be approximately half of that of the conjunctiva and approximately 10 times greater than that in the cornea.[21] It has been suggested that most of the drugs penetrating the sclera *in vivo* may be carried away by the blood circulation before diffusion to the intraocular tissues.[5,6] The high scleral permeability to drugs, as well as the conjunctival permeability, contributes to noncorneal ocular drug absorption.

## C. Intraocular Humors and Tissues

### *1. Aqueous Humor*

The cavity between the iris, chamber angle, and cornea is known as the anterior chamber, while that between the lens and iris–ciliary body is the posterior chamber. The total volume of these chambers in the human eye is typically 200–300 μL. Both cham-

bers are filled with aqueous humor, a fluid that provides nutrients and oxygen to the surrounding tissues while carrying waste away from them, thus maintaining the avascular cornea and lens transparency and assuring an optimal environment for light transmission. The aqueous humor is secreted by the ciliary processes into the posterior chamber and leaves the eye through the trabecular meshwork and Schlemm's canal at a flow rate of 2–5 μL/min.[25]

Elimination of drugs from the anterior chamber occurs most commonly by aqueous humor turnover. Generally, the clearance of ocular drugs is significantly greater than the aqueous humor turnover due to the metabolism and the systemic uptake by the vascular tissues of the uvea. The actual values of the ocular clearance of ophthalmic drugs vary from 4.7 μL/min to about 15 μL/min.[4] The apparent distribution volumes of ocular drugs have been reported as between 0.24 mL and 0.62 mL, but higher values have also been reported as a result of rapid tissue distribution.[4] The apparently small distribution volume of drugs suggests limited tissue distribution, possibly due to protein binding in the aqueous humor.

### 2. Iris–Ciliary Body

The ciliary body comprises the ciliary muscle and the ciliary processes. The outer epithelial cell layer facing the stroma of the processes is markedly pigmented. The iris consists of the pigmented epithelial cell layer, the iridial muscles, and the stroma. The binding of drugs with melanin in the pigmented tissue decreases the aqueous humor concentration of free drug and reduces the pharmacological response.[15] Accumulation of drugs in this pigmented tissue is also considered a source of ocular toxicity of drugs administered repeatedly by a topical or systemic route. Zane et al.[26] investigated the physicochemical factors associated with binding and retention of compounds in ocular melanin granules of rats. They demonstrated that multiple linear regression analysis is useful to predict the *in vivo* melanin binding of drugs associated with toxicity.

### 3. Lens

The lens has a refractive power of about 20 diopters and plays a very important role in the visual function of the eye. As it ages, the lens becomes more rigid and loses its refractive capacity. The lens is suspended by filaments, called zonules of Zinn, and is completely surrounded by a lens capsule consisting of an elastic collagenlike material. Proteins called crystallins make up more than 30% of the total weight of the lens, while the lens nucleus is composed of hard condensed cellular material that is expected to resist drug penetration. The lens, together with the pigmented tissues, may serve as a drug reservoir for the anterior chamber. However, accumulation of drugs in the lens has been known to induce cataract formation. Ahmed et al.[27] indicated

that the preferred route of penetration of timolol into the vitreous body was by diffusion around the epithelial capsule and the peripheral cortical layers of the lens.

### *4. Vitreous Body, Retina, and Choroid*

The vitreous body is located in the posterior cavity behind the lens. The vitreous body is a hydrogel of collagen and hyaluronic acid of high water content (> 98%). The turnover of water in the vitreous body has been reported to be about 10–15 min. Encircling the vitreous body is the retina, which functionally comprises the neural layer including photoreceptors, and the pigmented epithelium. Finally, the retina is itself encircled by the choroid and sclera, with the latter forming the outermost concentric ring of the globe. The choroid is a highly vascularized and pigmented tissue.

After topical instillation on the ocular surface, the access of drugs to the posterior segment is usually poor because there is little penetration through the crystalline lens or between the ciliary processes and crystalline lens. Drugs in the vitreous body are eliminated by the turnover of water in the vitreous body and by systemic uptake in the vascular tissues of the retina and choroid. Periocular injection of drugs may result in their penetration into the eye through the sclera, but the vascularized tissues will put most of the drugs into systemic circulation before they can reach the interior of the eye. To solve this problem, intravitreal drug delivery has been used for posterior segment disorders such as cytomegalovirus retinitis in patients with AIDS.[28] In this and similar cases, drug behavior in the vitreous body can first be analyzed by the diffusion model using the drug-concentration gradient found in the vitreous body.[29]

## D. Pharmacokinetics

Pharmacokinetics are important for maximizing the activity of drugs at the target tissues and organs. In the precorneal area, topically applied drugs are subject to competing processes, such as absorption in the ocular membranes, tear turnover and drainage, and enzymatic degradation.[30] Those drugs that make it through the precorneal area to penetrate the cornea first enter the aqueous humor and then are distributed to the iris–ciliary body, lens, vitreous body, and choroid-retina. Eventually they are eliminated by the aqueous humor flow and by uptake into the retinal blood flow. The pharmacokinetics of drugs in the eye can be analyzed using various compartment and physiological models, according to the characteristics of the drugs, and by considering the anatomical and physiological regions of the eye. For example, the compartment model has been used to describe the absorption behavior of topically applied fluorescein, pilocarpine, fluorometholone, timolol, and clonidine.[4,31-35] The statistical-moment theory has also been applied to describe the disposition of ethoxzolamide and its analogs applied topically to rabbit eyes.[36]

Figure 5 shows a typical 4-compartment model. The compartments consist of

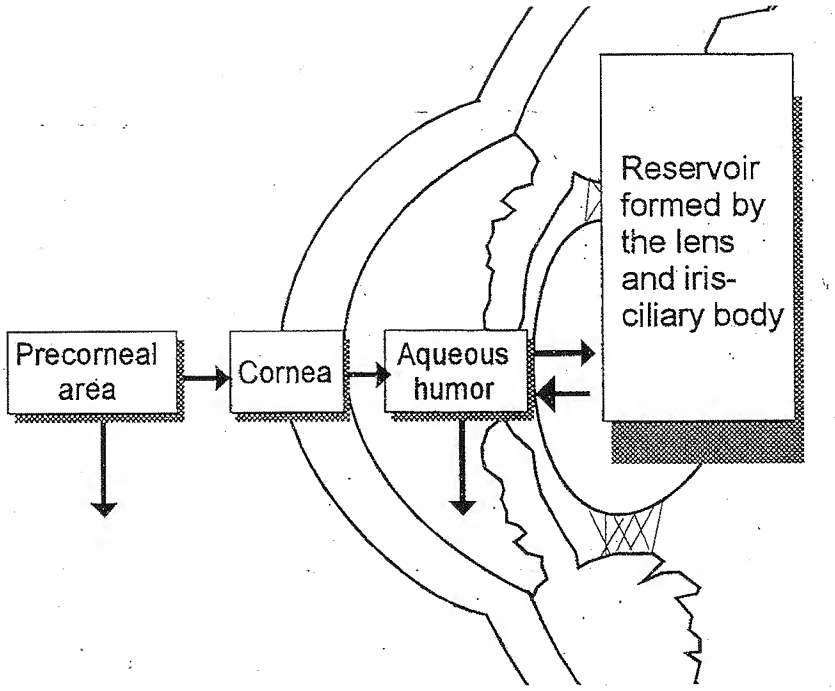
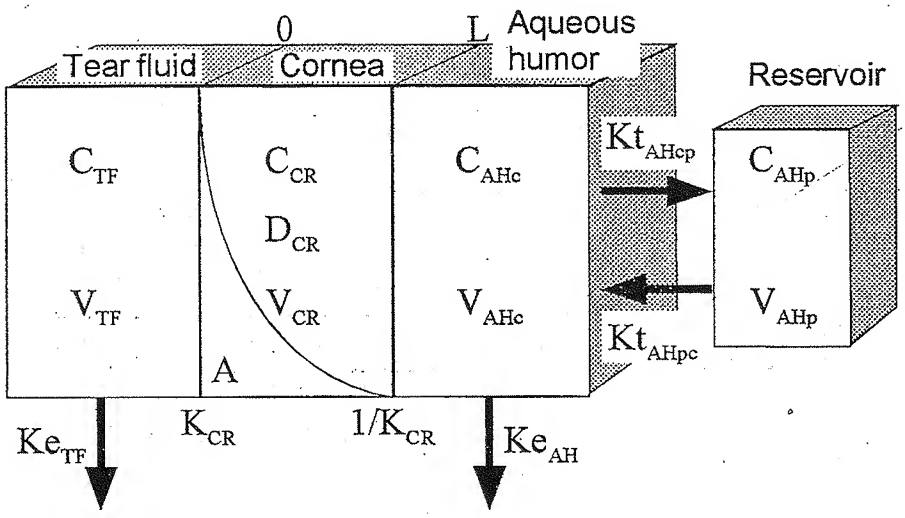



FIGURE 5. Pharmacokinetic model for ophthalmic drug after topical instillation on the ocular surface.

the precorneal area, the cornea, the aqueous humor, and the reservoir formed by the iris–ciliary body and the lens. Using this model, Makoid and Robinson[32] demonstrated an excellent correlation between predicted and observed values in multiple-dosing studies of pilocarpine. They found that the pilocarpine drainage rate at the precorneal area was one to two orders of magnitude larger than the corneal absorption-rate constant. The results from these studies clearly explain why only 1% to 2% of an administered dose of pilocarpine reaches the anterior chamber.

The computer program STELLA® (High Performance Systems, Lyme, NH) is useful for the simulation of drug behavior in complex compartment models. Grass and Lee[37] constructed a pharmacokinetic model using STELLA to predict the aqueous humor and plasma concentrations after topical instillation of timolol on the ocular surface. This program can be applied to any compartment model dealing with precorneal kinetics, nasal absorption kinetics, or plasma kinetics. Finne and Urtti[38] successfully applied STELLA to a simulation of timolol concentration in the eye after topical application of timolol in the form of polymer inserts.

Sugaya and Nagataki[39] measured changes in the pupil diameter in response to administration of various formulations of pilocarpine to the ocular surface, and ana-




$$Q_{TF}=X_0V_{TF}(V_{AHc}((s+Ke_{AH}+Kt_{AHcp})(s+Kt_{AHpc})-Kt_{AHcp}Kt_{AHpc})\sinh d + sZ(s+Kt_{AHpc})\cosh d)/W$$

$$Q_{CR}=X_0Z(V_{AHc}((s+Ke_{AH}+Kt_{AHcp})(s+Kt_{AHpc})-Kt_{AHcp}Kt_{AHpc})(\cosh d-1) + sZ(s+Kt_{AHpc})\sinh d)/W$$

$$Q_{AH}=sZX_0V_{AHc}(s+Kt_{AHpc})/W$$

$$W=V_{TF}V_{AHc}(s+Ke_{TF})((s+Ke_{AH}+Kt_{AHcp})(s+Kt_{AHpc})-Kt_{AHcp}Kt_{AHpc})\sinh d + sZV_{AHc}((s+Ke_{AH}+Kt_{AHcp})(s+Kt_{AHpc})-Kt_{AHcp}Kt_{AHpc})\cosh d + sZV_{TF}(s+Ke_{TF})(s+Kt_{AHpc})\cosh d + s^2Z^2(s+Kt_{AHpc})\sinh d$$

$$d=L(s/D_{CR})^{0.5}$$

$$Z=K_{CR}V_{CR}/d$$

**FIGURE 6.** Pharmacokinetic model including a diffusion process.Abbreviations: $Q_{TF}$, the Laplace transforms for the total amount of drug in the tear fluid; $Q_{CR}$, the Laplace transforms for the total amount of drug in the cornea; $Q_{AH}$, the Laplace transforms for the total amount of drug in the aqueous humor; s, Laplace operator with respect to time; $C_{TF}$, the drug concentration in the tear fluid; $C_{CR}$, the drug concentration in the cornea; $C_{AHc}$, the drug concentration in the aqueous humor; $C_{AHp}$, the drug concentration in the reservoir of the anterior chamber; $V_{TF}$, the apparent distribution volume in the tear fluid; $V_{CR}$, the corneal volume; $V_{AHc}$, the apparent distribution volume in the aqueous humor; $V_{AHp}$, the apparent distribution volume in the reservoir; $D_{CR}$, the diffusion coefficient of drug in the cornea; $K_{CR}$, the partition coefficient of drug between the cornea and tear fluid; A, the effective diffusion area; L, the effective diffusion length in the cornea; $Ke_{TF}$, the elimination rate constant in the tear fluid; $Ke_{AH}$, the elimination rate constant in the aqueous humor; $Kt_{AHcp}$, the transfer rate constant from the aqueous humor to the reservoir; $Kt_{AHpc}$, the transfer rate constant from the reservoir to the aqueous humor.

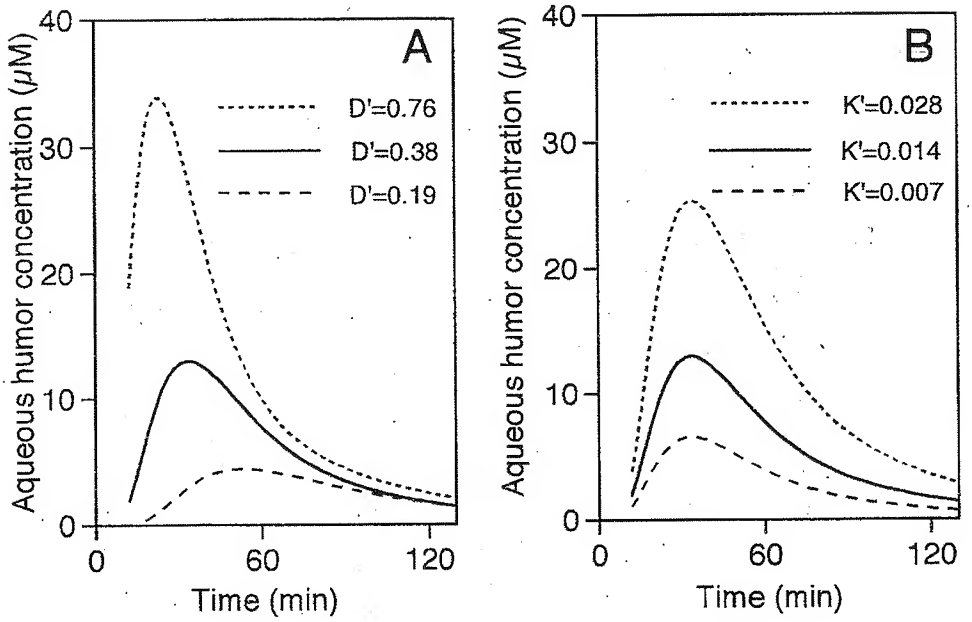



FIGURE 7. Simulation curve of timolol concentration in the aqueous humor after topical instillation in rabbit eyes according the pharmacokinetic model including a diffusion process (Figure 6). $D'$ ($D_{CR}/L^2$): diffusion parameter. $K'$ ($K_{CR}V_{CR}$): partition parameter. Control parameters for timolol ($D'$=0.38 $hr^{-1}$, $K'$=0.014 $cm^3$). (A) $D'$ changed. (B) $K'$ changed.

lyzed the response parameter time curve using a 2-compartment model. Pharmacological responses, such as pupil diameter and intraocular pressure, can thus be measured by noninvasive techniques. However, because of the many differences between species, great care should be taken in extrapolating the data on animals to applications for humans. And a further level of complexity is introduced when one attempts to extrapolate the data generated on normal eyes to the case of a pathological human eye.

Since the elimination of drugs in the precorneal area progresses under a non–steady-state and the corneal penetration of drugs is very slow compared to both the precorneal and the aqueous humor elimination of drugs, the process of corneal penetration of drugs may be best described by a diffusion model based on Fick's second law. Figure 6 provides a simple kinetic model representing the corneal diffusion process and the Laplace transform equation describing the drug concentration in the tear fluid, the cornea, and the aqueous humor. This model is based on the finite dose system that assumes one-plane membrane and first-order drug elimination from the tear fluid and the aqueous humor together with the lens and iris–ciliary body reservoir. This

model is useful to separately evaluate the diffusion and partition processes in the corneal penetration of drugs. Sasaki et al.[8] determined the *in vivo* parameters for timolol from drug concentrations in the tear fluid and aqueous humor after topical instillation in rabbit eyes. Figure 7A shows the influence of the corneal diffusion parameter on timolol concentration in the aqueous humor. An increase of the diffusion parameter enhances the maximum concentration and shortens the time to reach the maximum concentration. The diffusion parameter can be increased by destruction of the epithelial barrier, as it is in corneal ulcers and infections. Figure 7B shows that an increase of the corneal partition parameter enhances the maximum concentration of timolol in the aqueous humor. The cornea partition parameter can be improved by an increase of drug lipophilicity.

## III. MODIFICATION OF THE CORNEAL PENETRATION OF DRUGS BY CHEMICAL AND PHARMACEUTICAL APPROACHES

The penetration of drugs across the corneal epithelium occurs via transcellular and paracellular pathways. The former is a complex phenomenon that combines a passive contribution, membrane fixed charges, and an active contribution. In contrast, the latter represents the diffusive and convective transport occurring through intercellular spaces with tight junctions. A balance between the transcellular route and the paracellular route may characterize the membrane permeability of drugs. The first approach for enhancing corneal drug penetration is to modify the physicochemical property of the drug—mainly lipophilicity—by prodrug derivatization and ion-pair formation.

### A. Corneal Permeability

#### *1. Membrane Penetration*

The electrical resistance of a membrane is primarily a measure of ionic conductance through shunt or paracellular pathways consisting of tight junctions and intercellular spaces. Generally, epithelia with resistance in the range of 10–100 ohm $cm^2$ are considerably permeable, whereas those with resistance ranging from 300 ohm $cm^2$ to 10,000 ohm $cm^2$ are "tight" membranes. The cornea, which has a resistance of over 1,000 ohm $cm^2$, is a tight membrane compared to the intestine, nasal, bronchial, and tracheal membranes (200–300 ohm $cm^2$), but it is permeable compared to the stratum corneum of the skin (over 9,000 ohm $cm^2$).[40] The electrical conductances of various mucous membranes correlate to the steady-state fluxes of 6-carboxyfluorescein through the membranes. Klyce and Crosson[41] showed that the apical surface of the corneal epithelium alone contributed over half of the total electrical resistance of the

cornea. The top two layers are especially important as a barrier against hydrophilic drugs. This is due to the presence of annular tight junctions (zonula occludens), which completely surround and effectively seal the superficial cells of the epithelium to all but the smallest hydrophilic molecules. Thus, the cornea is impermeable to hydrophilic compounds because of a poor paracellular route due to the epithelial tight junctions. Accordingly, the transcellular route across the lipid cell membrane primarily contributes to the penetration of most ophthalmic drugs through the intact cornea.

The passive diffusion of drugs across the cornea is largely influenced by various factors, such as solubility, partition coefficient, molecular weight, and degree of ionization. In particular, the lipophilicity of the penetrant is one of the most important factors for cornea penetration. Multiple linear regression models have been applied to a series of β-blockers, naphthyl esters, and steroids.[17,42,43] These drugs had an almost parabolic relationship between the corneal permeability and the drug lipophilicity. The optimal partition coefficient for corneal drug penetration has been reported to be 2–3 in logarithmic values. This is consistent with the lipophilic nature of the corneal epithelium. A parabolic relationship has also been found to describe the influence of drug lipophilicity on corneal drug penetration of timolol prodrugs.[44] On the other hand, Wang et al.[45] demonstrated that a sigmoidal relationship, rather than the familiar parabolic relationship, best described the influence of lipophilicity on both conjunctival and corneal penetration of β-blockers.

Drug penetration through lipophilic membranes also follows the pH partition theory. Ionizable drugs are hydrophilic in the ionized form and lipophilic when unionized. Therefore, the pH affects the corneal permeability of weak acids and weak bases so that increasing the fraction of unionized molecules increases their lipophilicity and ocular absorption. Transcorneal penetration of free pilocarpine base has been found to be 2 to 3 times greater than that of the ionized form *in vitro*, and the miotic response of pilocarpine eye drops is reported to be greater at pH 7.0 than at pH 4.5.[46] On the other hand, in contrast to the corneal permeability, pH has shown only a small effect on the conjunctival permeability of atenolol, timolol, levobunolol, and betaxolol.[47] pH also increased the rate of the systemic absorption of topically applied timolol about 2.5 times by increasing the nasal and conjunctival permeabilities.[48] In general, it is difficult to predict the effect of pH on the overall drug absorption after topical instillation because pH irritation could cause reflex tears and corneal damage.

The corneal epithelium has a net negative charge at physiological pH because its isoelectric point is 3.2.[49] Accordingly, the corneal epithelium is selective to positively charged molecules. Liaw et al.[50] demonstrated a model of charge selective permeation in which the cornea was found to be 2 to 3 times more permeable to cations (L-lysine, benzylamine) than to anions (L-glutamic acid, salicylic acid). However, Mitra and Mikkelson[46] reported that the penetration of pilocarpine did not occur according to the pH partition theory. The ionized pilocarpine showed the same high rate of corneal penetration as the unionized pilocarpine. This could be explained by the formation of tightly bound ion pairs of pilocarpinium cations with dihydrogen phosphate or nitrate counter ions.

Eller et al.[51] evaluated an analog series representing modification to the benzene

ring of ethoxzolamide for solubility, pKa, partitioning, and permeability across isolated rabbit corneas. These physical parameters were correlated to Hammett sigma and Hansch pi parameter values for each compound. From these correlations, the authors developed a mathematical model relating the corneal permeability to molecular modifications of ethoxzolamide for the design of an optimum carbonic anhydrase inhibitor for topical use.

In addition to the passive diffusion of drugs via the paracellular pathway of the cornea, accumulated evidence in rabbit corneas indicates an active transport of ions. The active potential arises from an inward $Na^+$ transport from tears to aqueous humor and an outward $Cl^-$ transport in the reverse direction.[41] Through membrane active transport systems, the cornea generates a transepithelial potential that is negative on the epithelial side and positive on the endothelial side. Cooperstein[52] indicated that transport of aminoisobutyric acid is ouabain-sensitive and dependent upon the presence of extracellular $Na^+$ in the corneal epithelial cells of the toad. A stereo-specific carrier-mediated transport of L-lysine was also reported in rabbit corneas, with the L-lysine being cotransported with $Na^+$ and requiring a corresponding energy.[50] Generally, mucosal epithelial cells have various active transports for nutrients such as amino acids, dipeptides, fatty acid, and nucleotides. These carrier-mediated transports also participate in the transport of various other drugs.

## 2. Metabolism

Ocularly applied drugs are partly metabolized during or after corneal penetration. There are insufficient data for the estimation of the magnitude of this enzymatic barrier in the ocular tissues. However, there are significant levels of various enzymes in the ocular tissues. Many of these that may play a role in ocular drug metabolism have been identified, such as esterases, ketone reductase, catechol–O–methyltransferase, monoamine oxidase, acid phosphatase, steroid 6–ß–hydroxylase, oxidoreductase, aminopeptidases, UDP–glucuronyl transferase, arylsulfatase, glutathione conjugating enzymes, and aryl amine acetyltransferase.[53–55] In particular, the activity and penetration of peptide drugs may be decreased by the presence of significant levels of various peptidases and proteases at the delivery site and within the absorbing tissue. Among the anterior segment tissues, the corneal epithelium and the iris–ciliary body have been shown to have the most peptidase activity. In this regard, Lee et al.[55] showed a rapid enzymatic degradation of ocularly applied enkephalins.

In the case of ophthalmic prodrugs, their enzymatic conversion in the corneal epithelium can be utilized for releasing the active parent drug. The enzymatic conversion depends on a linkage structure between parent drug and pro-moiety. Among various linkage structures, esters are the most popular and have been investigated in detail in ophthalmology. Esterases are present in several ocular tissues, but not in the tear fluid. Esterase activity in the rabbit eye is highest in the iris–ciliary body followed by the cornea and the aqueous humor, with butyrylcholine esterase being the domi-

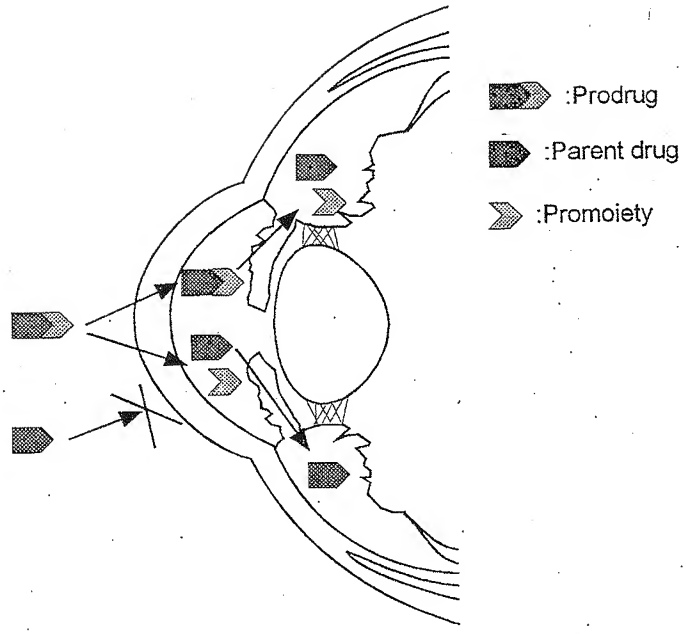



FIGURE 8. Schematic illustration of intraocular delivery of ophthalmic prodrug.

nant esterase in rabbit ocular tissues.[53] Another important enzyme is ketone reductase, which metabolizes a new antiglaucoma β-blocker, levobunolol, to active metabolite and retains the pharmacological effect. A high level of ketone reductase activity on levobunolol was found in the corneal epithelium and the iris–ciliary body of rabbits.[54] No activity was detected in the tear fluid, corneal stroma, sclera, or aqueous humor. The extent of levobunolol metabolism both during and after corneal penetration was dose-dependent. Ketone reductase is also important for site-specific activation of ketoxime derivatives of various β-blockers.[56–58]

In addition to the lipophilicity of prodrugs, their enzymatic lability is another factor that may influence the extent of improvement in corneal drug penetration by a prodrug. For example, the penetration of aliphatic timolol esters across the isolated rabbit cornea was found to depend on both lipophilicity and enzymatic lability.[44]

## B. Prodrug Approach

Prodrugs are defined as a pharmacologically inactive derivatives of drug molecules that are chemically or enzymatically converted to the active parent drugs (Figure 8). Most ophthalmic drugs contain functional groups such as alcohol, phenol, carboxylic

## TABLE 1
## Summary of Ophthalmic Prodrugs

| Parent drug | Prodrug modification | Animal | Effect | Reference |
|---|---|---|---|---|
| Steroid and epinephrine | | | | |
| Dexamethasone | Acetyl derivative | Rabbit | Improved efficacy in suppressing corneal inflammation | 64 |
| Prednisolone | Acetyl derivative | Rabbit | Improved efficacy in suppressing corneal inflammation | 65 |
| Epinephrine | Dipivalyl derivative | Human, rabbit | Increased corneal penetration 17 times with high lipophilicity and absorption 10 times | 61,66,67 |
| Beta-blocker | | | | |
| Timolol | Alkyl, cycloalkyl, and aryl derivative | Rabbit | Increased lipophilicity, corneal penetration, and ocular absorption, and improved therapeutic index | 44,73,77 |
| Timolol | Octanoyl, decanoyl, dodecanoyl, myristoyl, and palmitoyl derivative | Rabbit | Decreased corneal penetration but prolonged a significant ocular hypotensive activity | 80,81 |
| Atenolol, betaxolol, pindolol, propranolol, and timolol | (Acyloxy)alkyl carbamates | Rabbit | Increased corneal penetration 5 times for timolol and 8 times for pindolol | 74 |
| Nadolol | Diacetyl derivative | Rabbit | Increased absorption 10 times and ocular hypotensive activity 4 times | 35,72 |
| Terbutaline | Diisobutyryl derivative | Rabbit | Increased ocular hypotensive activity 100 times | 75 |
| Propranolol | O-acetyl, propionyl, butyryl, and pivaloyl derivative, and 2-oxazolidone derivative | Degradation experiments | Esters showed rapid regeneration of drug. Oxazolidone derivatives are too stable in buffer and plasma | 79 |
| Tilisolol | O-Acetyl, O-propionyl, O-butyryl, and O-valeryl derivative | Rabbit | Increased corneal penetration 3–6 times | 76 |

| Parent drug | Prodrug modification | Animal | Effect | Reference |
|---|---|---|---|---|
| Pilocarpine | | | | |
| Pilocarpine | Pilocarpic acid mono- and di-esters | Rabbit | Monoesters increased the duration of miotic response and diesters improved a stability and the miotic response of monoesters | 78,84,85, 86 |
| Pilocarpine | O, O'-(1,4-xylylene) bispilocarpic acid esters | Rabbit | Increased corneal penetration 2.3–7.2 times | 87,88,89, 90 |
| Phenylephrine | | | | |
| Phenylephrine | Pivalyl derivative | Rabbits | Increased mydriatic activity 15 times | 91 |
| Phenylephrine | Oxazolidine derivative | Rabbits, monkey | Increased ocular absorption 8 times and mydriatic activity 4 times | 92,93 |
| Carboxy anhydrase inhibitor | | | | |
| 6-Hydroxy-benzothiazole-2-sulfonamide | O-Acyl derivatives | Rabbit | Increased corneal penetration 40 times | 95 |
| Site specific delivery system | | | | |
| Propranolol, Timolol, Carteolol | Ketoxime derivatives | Rabbit, rat | Increased ocular hypotensive activity and decreased irritation | 56,57,58 |
| Alprenolol | Alprenoxime and alprenolon methoxime | Rat, rabbit | Showed ocular hypotensive activity and decreased systemic side effects | 97,98 |
| Adrenalone | Dibutyryl, dihexanoyl, diisovaleryl, di(ethyl succinyl), and diisovaleryl adrenalone | Rabbit | Showed ocular sympathomimetic activity without systemic acitivity | 99,100, 101 |
| Others | | | | |
| Prostaglandin $F_{2\alpha}$ | 13,14-Dihydro-17-phenyl-18,19,20-trinor-prostagrandin $F_{2\alpha}$ -1-isopropyl ester | Human | Showed ocular hypotensive activity clinically | 105 |

Continued

## TABLE 1
## Summary of Ophthalmic Prodrugs (continued)

| Parent drug | Prodrug modification | Animal | Effect | Reference |
|---|---|---|---|---|
| Prostaglandin $F_{2\alpha}$ | Acyl ester groups at the 9, 11, and 15 positions | Rabbit | 11 and 15-esters appears preferable for the design of $PGF_{2\alpha}$ prodrugs with potential clinical application. | 103 |
| Prostaglandin $F_{2\alpha}$ | 1-Isopropyl, 1, 11-lactone, 15-acetyl, 15-pivaloyl, 15-valeryl, and 11, 15-dipivaloyl derivatives | Rabbit | Increased corneal penetration 4-83 times | 106 |
| 5-Iodo-2'-deoxyuridine | Propionyl, butyryl, isobutyryl, valeryl, and pivaloyl derivative | Rabbit | Increased corneal penetration 1.5-3.1 times | 109 |
| Acyclovir | 2'-O-Glycyl derivative | Rabbit | Increased solubility about 30 times. Its 1% eyedrop was effective on stromal herpes simplex keratitis | 107 |
| Acyclovir | 2'-Aliphatic derivative | Rabbit | Increased lipophilicity and corneal penetration | 108 |
| 5-Fluorouracil | 1-Ethoxycarbonyl, 1-butyloxycarbonyl, 1-hexyloxycarbonyl, and 3-propionyl derivatives | Rabbit | Increased corneal penetration 10-50 times | 113 |
| Albuterol | Triacetyl, triisobutyryl, and tripivalyl derivatives | Rabbit | Showed ocular hypotensive activity | 114 |
| L-Carnosine | N$\alpha$-acetyl derivative | Rabbit | Increased ocular absorption significantly | 112 |
| Salicylate | Sodium monomethyl trisilanol orthohydroxybenzoate | Rabbit | Increased lens accumulation without conversion and itself showed aldose reductase inhibition | 110,111 |

acid, or amine that lend themselves to derivatization. An ideal ocular prodrug should be stable and soluble in aqueous solutions in order to enable formulation, sufficiently lipophilic in order to penetrate through the cornea, nonirritative, and capable of releasing the parent drug within the eye at a rate corresponding to the therapeutic need. In order to be useful, a prodrug must satisfy two criteria: it must have a linkage structure with adequate stability and lability and a promoiety with suitable physicochemical properties (lipophilicity, solubility, and pKa). Bodor[59] used a retrometabolism technique to develop a number of prodrugs. Currently existing ophthalmic prodrugs are summarized in Table 1.

Numerous prodrugs have been designed to improve the efficacy of ophthalmic drugs by enhancing their corneal penetration, prolonging their duration of action, and reducing their systemic and ocular side effects. Prodrugs have been tested experimentally and clinically in the eye, but stability and solubility problems, as well as local irritation, have limited their efficacy and clinical acceptability. The use of nonaqueous vehicles and acidic solutions has been shown to improve the instability of timolol prodrugs, but the usefulness of these prodrugs is still limited.[60] Coadministration of cyclodextrin increases the aqueous stability of ester prodrugs, and the chemical optimization of the prodrug structure is also useful for improvement of stability.[44,60]

Dipivalyl epinephrine, the first ophthalmic prodrug, was developed in the late 1970s to improve the corneal penetration of epinephrine, and remains the only commercially available ophthalmic prodrug.[61] Since then, many ophthalmic prodrugs have been reported for steroids, β-blockers, adrenergic agents, prostaglandins, and antiviral agents.[62,63]

### 1. Steroids and Dipivalylepinephrine

In clinical use, steroids are applied topically to treat inflammation of the anterior segment, although they have poor water solubility and membrane permeability. The acetate and the phosphate prodrugs of steroids were designed to improve corneal absorption and aqueous solubility. Acetyl esters of dexamethasone and prednisolone were found to increase the anti-inflammatory efficiency by a factor of 1.5–2.0 compared to their parent drugs.[64,65] These prodrugs are assumed to enzymatically convert to the parent drugs in the cornea, since both esterases and phosphatases are present in corneal tissue.

As another example, epinephrine (adrenaline), which reduces intraocular pressure and is clinically used as a topical antiglaucoma agent, is also characterized by a number of problems, including corneal irritation, cardiovascular side effects, short duration of action, and low bioavailability. Therefore, a lipophilic prodrug, dipivalyl epinephrine, was developed to reduce the application dose by increasing the bioavailability.[61,66] This prodrug is formed by pivalyl esterification of the hydroxyl groups on the epinephrine molecule and is used clinically in an acidic formulation because of its instability at physiological pH.[66,67] Dipivalyl epinephrine penetrates the human

cornea 17 times better than epinephrine because its partition coefficient is 100–600 times higher than that of the parent drug.[61] When administered in rabbits, the prodrug resulted in an approximately 10 times higher concentration of epinephrine in the aqueous humor than administration of free epinephrine, although the systemic absorption was similar in both cases.[68] With racemic dipivalyl epinephrine, 0.1% prodrug solution was as effective in lowering intraocular pressure as a 2% epinephrine solution. The associated systemic concentrations of epinephrine may be decreased by reduction of dose. And clinical studies have demonstrated fewer local adverse effects from dipivalyl epinephrine than from the parent drug.[69]

### 2. β-Blockers

β-Blockers decrease aqueous humor formation in the ciliary processes and are very often indispensable in the treatment of glaucoma.[70] However, because most of the topically applied β-blockers are rapidly absorbed into the systemic circulation, the usefulness of β-blockers in glaucoma has been limited by the relatively high incidence of cardiovascular and respiratory side effects.[70,71] Among the β-blockers examined as antiglaucoma agents, timolol, befunonol, carteolol, betaxolol, and levobunolol are used clinically. Also, prodrugs for various β-blockers have been reported to improve the ocular bioavailability.[72–76]

The nonselective β-blocker, timolol, is one of the most frequently prescribed drugs for glaucoma. Many prodrugs of timolol with varying lipophilicity have been developed and investigated in detail. In particular, alkyl, cycloalkyl, and aryl esters have been prepared by esterifying the hydroxyl group of timolol.[44,73,77] The original formulations of these prodrugs convert to timolol in the ocular tissues, and in physiological buffer at various rates. This instability problem was solved by utilizing sterically hindered esters, such as branched-chain and cycloalkyl esters, which are more stable than the straight-chain alkyl esters. The protonated amino group in the ester prodrugs made these drugs more susceptible to hydrolysis than the free base prodrugs.[78] Chien et al.[44] investigated the *in vitro* corneal and conjunctival penetration of a series of these esterified timolol prodrugs in rabbits and found that the corneal and conjunctival penetration of all prodrugs varied parabolically with their lipophilicity. The enzymatically more labile straight-chain alkyl esters penetrated the cornea more readily than the more stable branched-chain esters of comparable lipophilicity (Figure 9). The conjunctival permeability coefficients were not susceptible to the lipophilicities of prodrugs. The difference of ocular membranes in permeation character can control the extent and pathway for the ocular and systemic absorptions of prodrugs. Although the ocular absorption of timolol can be enhanced significantly by prodrug administration, its nearly complete systemic absorption cannot be increased. Actually, O-butylyl timolol increased the rabbit corneal absorption of timolol 4–6 times but did not affect the systemic absorption of timolol.[73] Consequently, the ratio of the aqueous humor concentration to the plasma concentration of timolol

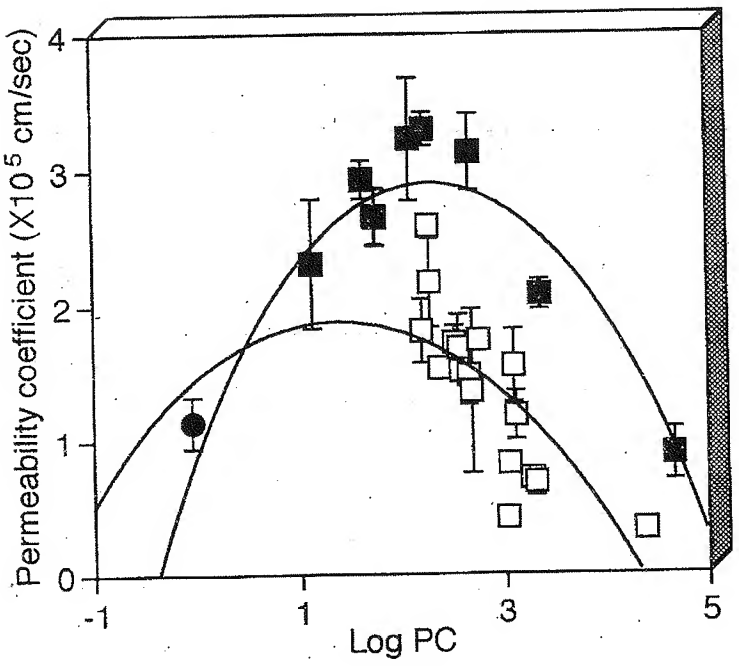



**FIGURE 9.** Influence of prodrug lipophilicity (log PC) on the corneal penetration of timolol ester prodrugs in the pigmented rabbit. Error bars represent standard deviation (n=4-6). Key: (●) timolol; (■) more labile prodrugs including standard-chain alkyl esters, O-cyclopropanoyl timolol, and O-1'-methylcyclopropanoyl timolol; (□) more stable prodrugs including all branched alkyl esters, all the cycloalkyl esters except those named above, and all the aryl esters. (From Chien, et al.,[44] with permission.)

in rabbit can be improved significantly by instillation of timolol prodrug rather than free timolol.

Tilisolol is another nonselective hydrophilic β-blocker for which lipophilic derivatives, O–acetyl, O–propionyl, O–butyryl, and O–valeryl esters, were synthesized.[76] These four derivatives showed increased lipophilicity and a rapid enzymatic conversion to tilisolol in the ocular tissue homogenates. The corneal penetration of the lipophilic derivatives was 3–6 times higher than that of tilisolol. Most of the drug that penetrated through the ocular membranes was detected as tilisolol, not as intact prodrug. Prodrugs also increased the concentration of tilisolol in the aqueous humor one hour after instillation in rabbits. The ratio of the area under the concentration time curve (AUC) in the aqueous humor to AUC in the plasma for tilisolol was enhanced by the prodrug instillation.

(Acyloxy)alkyl carbamates were developed as nonionic prodrugs bound to the amino

group of β-blockers such as atenolol, betaxolol, pindolol, propranolol, and timolol.[74] The enzyme-catalyzed hydrolytic reconversion to the free β-blocker in the plasma was very rapid and complete; however, the hydrolysis of the timolol prodrug in phosphate buffer was accompanied by the formation of significant quantities of oxazolidone that was completely resistant to the enzymatic cleavage to timolol in the rat plasma. On the other hand, no oxazolidone was detected in the buffer hydrolysis of the propranolol prodrug.[79] Increasing the lipophilicity of the prodrug increased the rabbit corneal permeability for pindolol and timolol by 8 and 5 times, respectively, but the effect was less significant on the more lipophilic prodrugs of betaxolol and propranolol.

Amphiphilic esters of timolol malonate have also been prepared, including octanoyl, decanoyl, dodecanoyl, myristoyl, and palmitoyl timolol.[80,81] The lipophilicity of these prodrugs was too high to allow them to penetrate the corneal epithelium. In fact, the timolol prodrug did not improve the *in vitro* corneal penetration compared with free timolol. However, the timolol prodrug decreased isoproterenol-induced ocular hypotension and exhibited a prolonged action in the intraocular pressure depression test. Although the mechanism of action was not clear, the authors attributed this pharmacological behavior to transscleral absorption or to accumulation of prodrug in the corneal epithelium.

### 3. Pilocarpine

Pilocarpine is a widely used drug for the treatment of chronic, simple, or wide-angle glaucoma, and acts by opening up the inefficient drainage channels in the trabecular meshwork by contraction or spasm of the ciliary muscle. Like acetylcholine, another cholinergic muscarinic agonist, pilocarpine, contracts the ciliary muscle that is cholinergically innervated with nerve fibers. However, pilocarpine shows poor bioavailability and short duration of action after its topical application. Dosing of pilocarpine 3–6 times a day leads to transient high concentration in the eye and results in dose-related side effects such as myopia, miosis, and headache. Various pharmaceutical approaches, such as viscous vehicles, latices, emulsions, and presoaked hydrogel contact lenses have been developed to prolong the duration of action of pilocarpine.[3] The rate-controlled pilocarpine delivery system, Ocusert®, was developed by Alza Corporation (Palo Alto, CA) as an ocular insert for human glaucoma treatment.[82,83]

Monoester derivatives of pilocarpine were developed to improve the ocular bioavailability of this drug.[84–86] Pilocarpine was regenerated from the monoesters by the spontaneous cyclization of the lactone ring via intramolecular nucleophilic attack on the ester carbonyl, without the assistance of enzymes. The monoesters were more lipophilic than the free pilocarpine and increased the ocular bioavailability, as determined by miosis. Prolonged duration of the pilocarpine action was also observed in several monoesters. However, the instability of the monoesters by the lactonization in an aqueous solution limited their utility. The rate of cyclization increased with increasing pH and temperature, but the monoesters were found to be more stable at an acidic

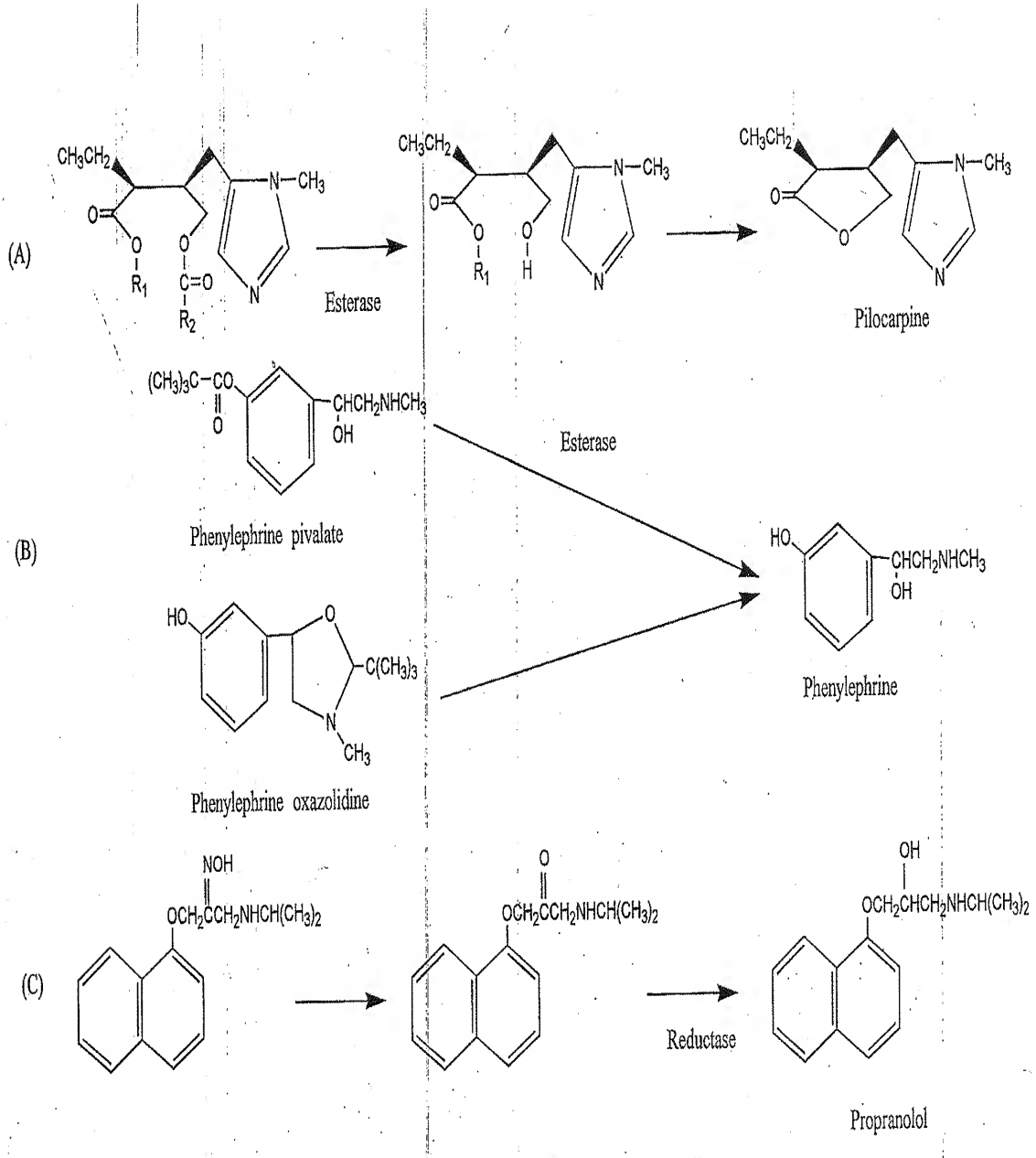



**FIGURE 10.** Regeneration process of (A) pilocarpine, (B) phenylephrine, and (C) propranolol from their prodrugs.

pH than at physiological pH. However, the application of an acidic solution on the eye causes irritation.

Diester prodrugs (double prodrugs) were developed to solve the stability problem of the monoesters.[84–86] In the double prodrugs, an enzymatic release mechanism is required prior to the spontaneous cyclization for regeneration of the pilocarpine (Figure 10A). Diesters were more stable and had the additional advantage of being more lipophilic than either free pilocarpine or the pilocarpine monoesters. In rabbits, the action of monoesters has been shown to be prolonged by a factor of 1.5 as compared to that of pilocarpine, while the action of diesters improved by a factor of 2.3 as determined by miosis.

A series of bispilocarpic acid mono- and diesters were developed as dimeric pilocarpine prodrugs with two pilocarpine units attached via a hydrocarbon chain to minimize the amount of promoieties.[87–89] Bispilocarpic acid monoesters cyclized spontaneously to pilocarpine via intramolecular nucleophilic attack. According to the double prodrug concept, diester prodrugs were also prepared to solve the stability problem of monoester. In this preparation, a single prodrug molecule was hydrolyzed by an esterase to produce two pilocarpines molecules. Despite their large molecular weights, these prodrugs showed 2–7 times higher permeability than free pilocarpine through an excised cornea of rabbits, and were observed only as free pilocarpine on the endothelial side. A parabolic relationship between the apparent partition coefficient and corneal permeability has been observed. However, significant eye irritation was reported to occur immediately after administration of pilocarpine prodrugs in the form of eye drops. Addition of cyclodextrin to the formulation increased the stability and decreased the eye irritation by pilocarpine prodrugs.[90] However, the complex of cyclodextrin and prodrug probably reduces the corneal absorption of prodrug in addition to increasing the prodrug solubility on the precorneal tear film.

### *4. Phenylephrine*

Phenylephrine is an α-adrenergic agent that is routinely used for mydriasis during cataract surgery and ophthalmoscopic examination. However, phenylephrine is another drug with poor corneal penetration and with side effects such as corneal irritation, transient hypertension, and adverse cardiovascular effects. The pivalate ester and oxazolidine prodrugs have been investigated as a means of improving the bioavailability of phenylephrine (Figure 10B). The pivalate ester was about 10 times more potent than the free phenylephrine in terms of inducing mydriatic activity in rabbits, but the prodrug itself was reported to possess the intrinsic activity.[91] The oxazolidine prodrug showed a 1,000-fold increase in partition coefficient compared with phenylephrine,[92] and converted to phenylephrine very rapidly in aqueous solution. The oxazolidine prodrug eye drops were therefore formulated in sesame oil. The pharmacokinetics of topically applied phenylephrine were then estimated from the induced mydriatic activity. In rabbits, monkeys, and human volunteers, topical administration

of 1% prodrug produced a rate of mydriasis approximately equal to that produced by 10% phenylephrine hydrochloride, thereby substantially reducing the potential for cardiovascular effects.[93]

### 5. Carbonic Anhydrase Inhibitors

Carbonic anhydrase inhibitors, acetazolamide, methazolamide, dichlorophenamide, and ethoxzolamide, have been administered orally for treatment of glaucoma due to their ability to lower intraocular pressure by reducing the aqueous humor formation. However, inhibition of carbonic anhydrase in the extraocular tissues results in undesirable side effects such as paresthesia, malaise, fatigue, depression, anorexia, and nausea. The effect of topical application of carbonic anhydrase inhibitor on the localization of the drug in the ciliary process and on the systemic side effects has been investigated. In clinical trials, however, carbonic anhydrase inhibitors applied topically to the eye have demonstrated little ability to reduce intraocular pressure.[94] The ineffectiveness of these drugs is probably due to their poor aqueous solubility and poor permeability through the cornea.

Various carbonic anhydrase inhibitors, including prodrugs for topical use, have been developed by chemical derivatization.[36,51] Woltersdorf et al.[95] prepared a series of O-acyl derivatives of 6-hydroxybenzothiazole-2-sulfonamide for topical application to improve ocular delivery of carbonic anhydrases. Most derivatives regenerated the active parent drug by esterase hydrolysis during corneal transport. Among them, the 2-sulfamoyl-6-benzothiazolyl 2,2-dimethylpropionate prodrug was found to enhance drug delivery through the isolated rabbit cornea by a factor of 40 over that of the parent active substance, and successfully decreased the intraocular pressure after topical instillation. Larsen et al.[96] also reported the water-soluble amino-acid derivatives of N-methylsulfonamides as candidates for topically-active carbonic anhydrase inhibitors.

### 6. Site-Specific Delivery

Bodor et al.[56] have developed ketoxime derivatives of β-blockers as delivery systems for targeting ophthalmic drugs within the eye that were evaluated by predictable site-specific metabolisms such as that of ketone reductase in the iris-ciliary body. Weight-for-weight, the iris-ciliary body is the most enzymatically active organ in the body. Ketone reductase is present mainly in the corneal epithelium and the iris-ciliary body, where the ketoxime is hydrolyzed to produce the ketone form. The ketone form is then enzymatically reduced to its parent β-blocker in the iris-ciliary body (Figure 10C). Among the ketoximes of propranolol, timolol, and carteolol, the propranolol ketoxime is more effective and much less of an irritant compared to its parent β-blocker. While the ketoximes also displayed activity on isoprenaline-induced tachycardia after intra-

venous administration, they were devoid of activity when given orally. Propranolol was present for a prolonged period and in significant concentrations in various ocular tissues of rabbits after instillation of its ketoxime precursor. In rats, however, propranolol was not detected in blood following systemic administration of the oxime.[57] One reason for the high activity of the ketoxime derivatives of β-blockers in decreasing intraocular pressure in rabbits may be the stereospecificity of the ketone-reduction step since, after the instillation of the racemic propranolol oxime, only the active S-(-)-form of propranolol was detected in the cornea and the iris–ciliary body.[58] Timolone oxime has also shown significant ocular hypotensive activity, which was faster in its onset and shorter in its duration than that of timolol itself. However, ketoxime derivatives of the isopropyl analog of timolol and carteolol did not show any action on the intraocular pressure of rabbits.

Another ketoxime derivative of the β-blocker alprenoxime was designed and investigated as a potential novel antiglaucoma agent.[97] Alprenoxime produced significant reduction of the intraocular pressure starting at 30 min and lasting for more than six hours after topical administration in rabbits. In both rats and rabbits, the intravenous injection of alprenoxime led to insignificant transient bradycardia, while no activity was found after oral or topical administration. In contrast, alprenolol in a similar dose produced sustained and significant bradycardia for more than 30 min. Alprenoxime also showed negligible β-adrenergic blocking activity when assessed against isoprenaline-induced tachycardia. These results indicate that the drug has potent ocular hypotensive action and weak systemic β-adrenergic blocking and cardiovascular activity. Alprenoxime, however, has low stability and is a mild irritant to the eye. Intramolecular H-bonding of the hydroxyimino group possibly contributes to the hydrolysis of the oxime analog. Therefore, the methoxyimino function was introduced for the transient protection of the chemically unstable ketone to improved solution stability by a factor of 1.7, when only 10% of the alprenolone methoxime was degraded, compared to the oxime analog.[98] Also, the topical use of the methoxime analog resulted in significant and prolonged decrease of the intraocular pressure in rabbits.

Diesters of the inactive ketone analog (adrenalone) of epinephrine have also been developed as site-specific delivery systems that were converted to epinephrine in the iris–ciliary body.[99–101] Many of these diesters of adrenalone have high ocular sympathomimetic activity despite the fact that adrenalone itself, even if delivered intraocularly, is practically inactive. The mechanism of action of these adrenalone derivatives was found to be due to the formation of adrenaline from the diester, but not from adrenalone. While the inactive adrenalone is also formed hydrolytically and found in every compartment of the eye, the active adrenaline was found only in the iris–ciliary body, due to a reduction-hydrolytic steps sequence.[100] The marked differences in the ratio of ketone reductase to esterase levels among the anterior segment tissues causes the site-specific delivery of diesters. Also, the susceptibility of the ester linkage to hydrolysis determines the duration of the action of the diesters, as shown by the diethylsuccinyl adrenalone, which has a plasma half life of 1 min. and is anticipated to have

a shorter duration of action than the divaleryl adrenalone, which has a plasma halflife of 58 min.[101]

Bodor[59] has demonstrated that the soft drug approach is the most useful for designing safe and selective ophthalmic drugs. Using the soft drug approach, active drugs that rapidly undergo predictable enzymatic inactivation (soft drug) have been developed by chemical modification from known inactive metabolites of ophthalmic drugs. Based on this concept, soft anticholinergics that show short mydriatic cycloplegic activity and soft corticosteroids that show sufficient anti-inflammatory activity in the eye were developed.[59] Some soft drugs, such as tematropium methyl bromide, adaprolol maleate, and loteprednol etabonate, have reached various stages of development for human use.

### 7. Others

Prostaglandin $F_{2\alpha}$ has been investigated to bring about a long-lasting and highly significant decrease in intraocular pressure in primates by increasing the uveoscleral outflow of the aqueous humor. However, the disadvantages of the use of prostaglandin derivatives in the eye are initial hypertension and inflammatory responses. Various prostaglandin derivatives for topical use have been developed by chemical derivatization, including the prodrug approach, for the treatment of glaucoma to improve the reduction of intraocular pressure and to decrease side effects associated with the prostaglandins' autacoid activity.[102] Using the prodrug approach, methyl, ethyl, and isopropyl ester prodrugs of prostaglandin $F_{2\alpha}$ were designed to improve its corneal penetration.[103] Esterification of the carboxyl group of prostaglandin $F_{2\alpha}$ increases the ocular hypotensive potency by enhancing the penetration of the drug into the eye. These prodrugs have also been reported to be less irritating, with the additional advantage of a 10 to 30 times gain in intraocular-pressure–reduction potency compared to the parent drug. Inserting a benzene ring in the $\omega$ chain of prostaglandin $F_{2\alpha}$ greatly alters the potency and receptor profile of prostaglandin $F_{2\alpha}$.[104] This prostaglandin $F_{2\alpha}$ analog (latanoprost), which is highly selective for the F-type prostaglandin receptor, has been launched on the market as a potent and selective intraocular-pressure–reducing agent.[105] Chien et al.[106] evaluated the rabbit corneal penetration of various prodrugs of prostaglandin $F_{2\alpha}$ and demonstrated no direct correlation between the drugs' lipophilicity and corneal permeability. Among them, the 1,11–lactone and 11,15–dipivaloyl ester prodrugs appeared to be superior to the others in providing bioavailable prostaglandin $F_{2\alpha}$ for ocular hypotension, while minimizing hyperemia.

Acyclovir is a potent and selective antiherpes drug with a marked activity against *herpes simplex* virus (HSV) types 1 and 2 and *varicella zoster* virus. The efficacy of ocular ointment of 3% acyclovir is well established for the treatment of herpetic epithelial keratitis. Acyclovir, however, can be used only in ointment form because of its poor aqueous solubility, and ointments have the disadvantage of blurring the

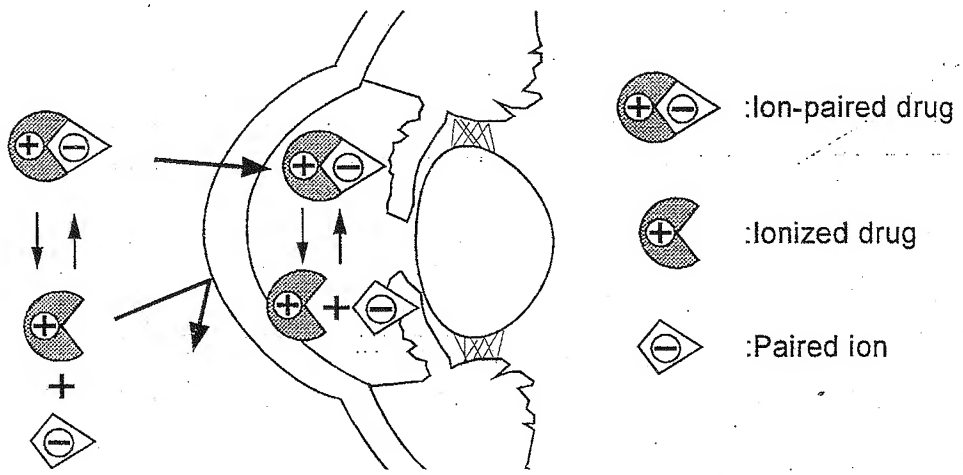



FIGURE 11. Schematic illustration of ophthalmic lipophilic ion pair.

vision and creating a greasy, sticky sensation in the eye. Accordingly, 2′-O-glycylacyclovir was developed to improve the aqueous solubility of this drug. This water-soluble ester of acyclovir can be prescribed in the form of eye drops at a concentration that would not be feasible for acyclovir itself.[107] 2′-O-Glycylacyclovir (1%) has been shown to significantly reduce the severity of several HSV eye disease manifestations, such as epithelial and stromal keratitis and iritis. Lipophilic aliphatic 2′-esters of acyclovir have also been developed to increase the ocular bioavailability of acyclovir.[108] These prodrugs were hydrolyzed to acyclovir in ocular tissue homogenates and showed increase corneal penetration with increasing lipophilicity of the esters.

5-Iodo-2′-deoxyuridine (idoxuridine) is a halogenated pyrimidine derivative used for the topical treatment of *herpes simplex* keratitis. However, because of its polar nature idoxuridine permeates poorly into the cornea. The poor bioavailability of this drug results in a high incidence of treatment failure unrelated to the resistance of viral strains. Therefore, five aliphatic prodrugs of idoxuridine were synthesized for potential use in the treatment of deep ocular infections such as stromal keratitis, iritis, and retinitis.[109] A parabolic relationship was found between the *in vitro* corneal permeability and the carbon chain length of the prodrugs. Enzymatic hydrolysis proceeded most readily in the iris-ciliary body, followed by the cornea and aqueous humor. Among the prodrugs, the 5′-butyryl ester exhibited an approx-

imately four-fold increase in aqueous humor concentration compared to idoxuridine after instillation in rabbits.

Since cataract is the most frequent cause of human blindness in the world, the prodrug approach has also been used for development of anticataract agents, such as aldose reductase inhibitors, salicylates, and antioxidants.[110-112] Ophthalmic prodrugs of the antimetabolite, 5-fluorouracil, and β-adrenergic agonist, albuterol, have also been developed.[113,114]

## C. Ion Pairs

Lipophilic ion-pair formation is another approach to enhancing the penetration of drugs through the intact cornea, in this case by intermolecular electrical interaction between drugs and counterions (Figure 11).[115] To operate in this manner, counterions require several properties, such as high lipophilicity, sufficient stability, physiological compatibility, and metabolic stability. The ability of ion-pair formation to influence the behavior of drugs depends strongly on the physicochemical properties of both the drugs and the counterions.

The concept of the ion pair was first introduced to explain the decrease in electrical conductance of sodium chloride in liquid ammonia. An ophthalmic ion-pair formation was reported between cromolyn sodium as an organic anion and benzalkonium chloride as an organic cation.[116] The extent and rate of corneal penetration of these two large ions was improved upon their coadministration. Although this example indicates the possibility of ion pairing, the concept of ion-pair transport is still not extensively applied and some authors have reported that the penetration of certain drugs was not affected by ion-pair formation.[117] Lipophilic anions and cations used as counterions are often reported to enhance drug penetration through the cornea by affecting the structure of the biological membrane. On the other hand, Hadgraft et al.[118] demonstrated that ion-pair formation of penetration enhancer partly contributes to its enhancing effect.

The formation of an ion pair was demonstrated between bunazosin as an organic cation and anious fatty acids as the organic anion.[119,120] The extent of drug ionization, hence the pH of the medium and the pKa of the drug, and the chain length of fatty acid were both important factors determining the extent of improvement in the ocular ion-pair penetration. Among various fatty acids, capric acid significantly increased both the permeability of the bunazosin ion pair and the partition of bunazosin in the eye. Both of these enhancements were maximal when the molar ratio of bunazosin to caprylic acid was unity. The bunazosin permeability was not increased by pretreatment of the cornea with caprylic acid. Nor did caprylic acid affect the permeability of non-ionic methylparaben through the cornea or the interaction of bunazosin with protein. These results strongly suggest the importance of ion-pair formation in the ability of caprylic acid to enhance corneal permeability.

## IV. MODIFICATION OF THE OCULAR MEMBRANES BY A PHARMACEUTICAL APPROACH

The second approach to improving the ocular permeability of drugs is to modify the integrity of the corneal epithelium transiently by a pharmaceutical approach. This can be accomplished by exposing the eye to amphiphilic substances and chelating agents. The enhancing effect of most penetration enhancers has been correlated with the histological damage to the biological membrane. Each enhancer must be evaluated in relation to its potential use and with regard to long-term safety and toxicological considerations. In order to use penetration enhancers with safety, further information is needed on the mechanism of regulation between the transcellular membrane and paracellular tight junction.

### A. Penetration and Regulatory Pathways

#### *1. Transcellular Penetration*

Epithelial cells are surrounded by an outer cell membrane composed of a phospholipid bilayer. The polar heads of the phospholipids are at the surfaces of the membrane, and the nonpolar tails make up the membrane's interior. Protein molecules are embedded in the lipid membrane. The fluid-mosaic model, a working description of membrane structure, suggests that the pattern of protein molecules within the phospholipid bilayer is in flux. The lipids in the cell membranes are heterogeneous, and consist mainly of phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, the simple glycolipids glucosylceramide and galactosylceramide, and cholesterol.

The composition of the acylchains of the phospholipids and glycolipids is heterogeneous with respect to both chain length and degree of unsaturation. Phosphatidylcholine shows various polymorphic states in aqueous solution, such as the "gel" (rigid chain) and "liquid crystalline" (fluid chain) states. Transition between the gel state and liquid crystalline state occurs at the lipid-specific transition temperature. The apical membrane is rich in glycolipids and cholesterol. Glycolipids, because of their high transition temperature, provide stabilization at body temperature, while cholesterol, because it is a rigid molecule, has the effect of making a liquid crystalline bilayer less fluid. Cell membranes must maintain impermeability to small inorganic ions in order to produce the transmembrane ion gradients. The active transports of various nutrients are driven by these ion gradients.

The common phospholipids are insoluble but swelling amphiphilic substances. These substances aggregate in bilayer sheets of effectively infinite dimensions to compose a lipid membrane. Penetration enhancers (bile salts, benzalkonium chloride, sodium dodecyl sulfate, and Triton X-100) are soluble amphiphilic compounds that mostly exhibit significant surface activity. In their role as penetration enhancers, the surfac-

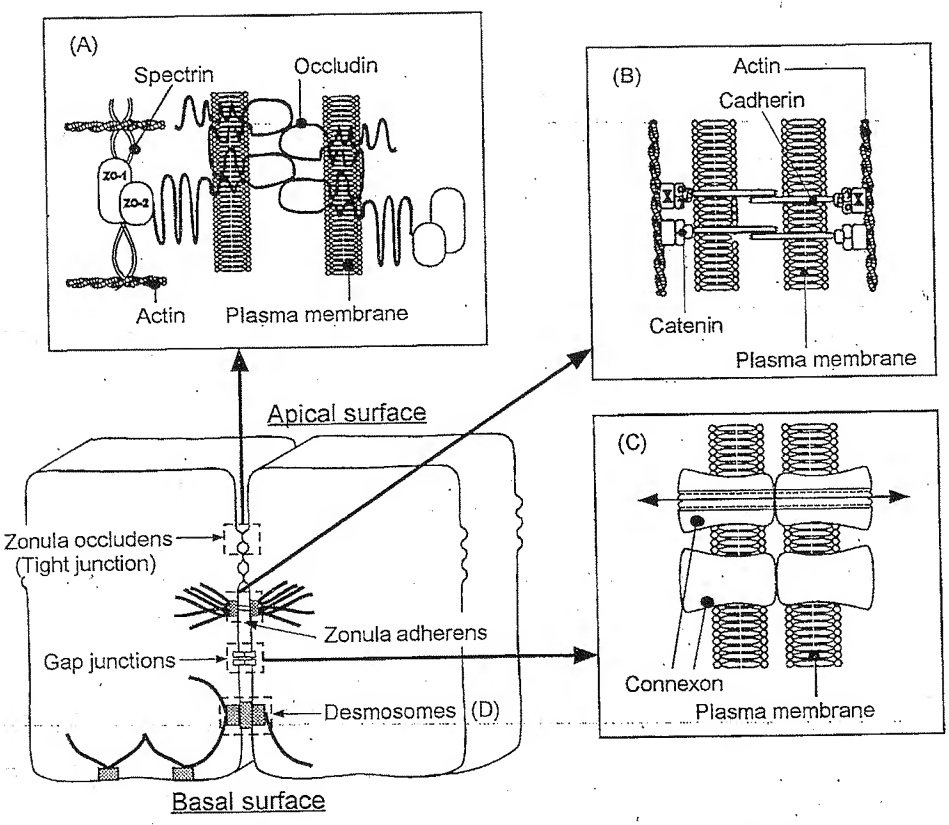



FIGURE 12. Schematic illustration of intercellular junction in epithelial cells. (A) Tight junction; (B) adherens junctions; (C) gap junctions; (D) desmosomes.

tants have aqueous solubilities that can be in the millimolar range, much higher than the aqueous solubilities of membrane phospholipids. The surfactants can thus partition into the cell membrane, where they can form polar defects in the lipid bilayer. This disruption and fluidization of the ordered lipid layer results in an increased penetration of drugs. At high surfactant concentrations in the cell membrane, surfactant molecules contact each other and cause the membrane to dissolve into surfactant-membrane mixed micelles. It is also well known that surfactants can extract proteins from the plasma membrane of the cells. These physical effects of surfactants on the cell membrane are nonspecific and increase the permeability of the membrane to both drugs and ions.[121] Because the dispersion of ion gradients can affect the viability of the cell, at high concentration of surfactants the epithelial cells might undergo irreversible lysis and thereby allow macromolecules to penetrate the epithelial membrane. The dispersion of ion gradients also influences the cell cytoskeleton, which in turn plays a role in forming the tight junctions.

Several physical techniques, such as differential scanning colorimetry, X-ray diffraction, Fourier transform infrared spectroscopy, nuclear magnetic resonance, and electron spin resonance spectroscopy, have been used to clarify the interaction of drug-penetration enhancers with biological membranes and artificial lipid membranes. From these studies it is known that the drug-penetration enhancers strongly interact with membrane lipids and proteins to increase transcellular penetration of drugs. A good correlation between reduced nonprotein thiols and enhanced penetration of hydrophilic compounds by diethyl maleate and salicylates has also been reported.[122] Some drug-penetration enhancers have been found to possess not only the enhancing effect but also an inhibitory effect on drug metabolism, both of which may contribute to the penetration enhancement of drugs, and particularly peptide drugs.[123]

### 2. Paracellular Penetration

Anatomically, the paracellular pathway consists of the intercellular space with intercellular junctions. The intercellular junctions are categorized into four types: adherens junctions, desmosomes, gap junctions, and tight junctions (Figure 12),[124,125] which were originally identified and defined by electron microscopy. Adherens junctions are formed by homotypic association of the transmembrane protein cadherin, which associates directly with the cytoplasmic proteins α-, β-, and γ-catenin.[126] Cadherins are a family of glycoproteins that is responsible for $Ca^{2+}$-dependent cell–cell adhesion and that includes E–cadherin/uvomorulin, N–cadherin/A–CAM, P–cadherin, L–CAM, and several other cadherins. Desmosomes are structures that bind cells to each other. They are not thought to be a barrier for drug penetration because they are separated by a distance of approximately 30 nm. The desmosomal integral membrane proteins are called desmogleins and desmocollins.[127] Gap junctions include connexons that are constructed from six connexins and that contain porous channels considered to be important for communication between cells.[128] Because molecules of less than 1,000 Da can permeate the pores of the connexons, the connexons are not thought to be barriers for drug penetration.

The role of cell–cell junctional spaces in drug penetration has been investigated by observation of the movement of horseradish peroxidase (molecular weight 40,000) through the rabbit cornea.[129] In this study, horseradish peroxidase was injected into the anterior chamber and its progress through the cornea was monitored using immunocytochemical staining. Horseradish peroxidase was found to readily penetrate the corneal endothelium and stroma as well as the bottom three to four layers of the corneal epithelium. The intercellular space in the basal layer of the epithelium is rather wide and is permeable to horseradish peroxidase. However, horseradish peroxidase permeation through the cornea stopped within the top two to three epithelial cell layers due to tight junctions between the cells. These tight junctions are the main barrier to the transepithelial penetration of ions and neutral molecules through the paracellular pathway.

Tight junctions are band-like regions between the membranes of adjacent epithelial cells that appear to contact each other, and completely encircle the superficial epithelial cells.[124,125] The tight junctions are also considered to function as the boundary between the apical and basolateral domains in the plasma membrane that results in the polarization of the cell. The polarized distribution of ion channels, pumps, and enzymes into the apical and basolateral domains is responsible for the vectorial nature of the transepithelial transport.

The tight junction related protein ZO-1 has been obtained from purified mouse biliary ducts,[130] and has been used as a marker of tight junctions. Several related proteins, such as cingulin (140, 108 kD), 7H6 antigen (155 kD), p130 (130kD), and ZO-2 (160 kD), were also identified using monoclonal antibody prepared from ZO-1. Recently, the transmembrane protein, occludin, was identified in the tight junction.[131] The homotypic contacts of the occludin form the intercellular barrier at the tight junction. The occludin is bound directly to ZO-1 on the cytoplasmic surface. Analysis of occludin cDNA has indicated that the occludin molecule consists of four transmembrane segments with both $-NH_2$ and $-COOH$ terminals exposed at the cytoplasmic surface.

The actin-binding proteins, vinculin and α-actinin, have been localized in the cytoplasmic region adjacent of the tight junction. These cytoplasmic proteins probably play an important role in organizing the paracellular seal. Ultrastructural evidence also indicates that microfilaments and microtubules exhibit a close association with cellular junctions.[132] The cytoskeleton is a complex protein network made up of microfilaments, intermediate filaments, and microtubules, and is responsible for such functions as mechanical strength, cell adhesion, and cell motility. In the vicinity of the tight junctions the cytoskeleton forms a ring completely encircling the cytoplasm of the cell (perijunctional actomyosin ring). The ring can be visualized by staining filamentous actin with fluorescein-labeled phalloidin. Global changes in actin distribution can be directly determined by using any of several tight junction disrupting agents as markers.[133]

Although the regulatory mechanism of the tight junctions is not well understood, probably both the cellular $Ca^{2+}$ levels and the actin filaments of the cytoskeleton play important roles in the integrity of the tight junctions. Understanding the mechanisms of tight junction regulators may be useful for the modulation of tight junctions in a reversible manner. Treatment with the cytoskeletal modulators, cytochalasins and colchicine, or removal of the extracellular $Ca^{2+}$ has been found to increase the tight junction permeability.[134] Both the assembly and barrier properties of tight junctions are also influenced by all the classic second messenger and signaling pathways, including tyrosine kinases, $Ca^{2+}$, protein kinase C, G proteins, calmodulin, adenosine 3′,5′-cyclic monophosphate, and phospholipase C. The effects are not always consistent among cell types, suggesting the possibility that different regulatory mechanisms operate or predominate in different cell types.[133]

Recent research suggests that many surfactants increase the paracellular penetration of drugs by affecting the tight junctions.[135-137] Palmitoylcarnitine, a zwitterionic surfactant, has been shown to enhance drug absorption without causing signif-

icant changes to the morphology of intestinal cells both *in vivo* and *in vitro*, suggesting that this surfactant increases the paracellular penetration via the tight junctions.[136] Disruption of tight junction integrity has also been reported using other surfactants, such as sodium caprate and sodium dodecyl sulfate.[137]

Madara and Pappenheimer[138] demonstrated that the paracellular permeability of rodent small intestine was enhanced by glucose and other nutrients, and proposed this enhancement as a mechanism to augment the absorption of nutrient solutes through paracellular solvent drag. An accompanying morphological correlate of the enhanced permeability was the aggregation of the perijunctional actin filaments. In other words, the active transport of glucose or amino acids, which is coupled to $Na^+$ transport, across the intestinal mucosa into the intercellular lateral spaces creates an osmotic force for fluid flow, and triggers contraction of the perijunctional actomyosin ring that results in increased paracellular permeability. Also, Martinez–Palomo and Erlij[139] showed that solutions made hypertonic with lysine induce a reversible opening to the tight junction of the toad urinary bladder.

## B. Penetration Enhancers

The use of penetration enhancers could be advantageous with most drugs, and could be convenient in the manufacturing of ophthalmic preparations. Although penetration enhancers have been extensively studied in percutaneous, nasal, and buccal delivery of drugs, there have been few studies on the penetration enhancement of ocular drugs because of the sensitivity of the eye to the enhancers. Penetration enhancers are required to be pharmacologically inert and chemically stable, to have a high degree of potency in terms of both specific activity and reversible effects on cornea permeability, and to be both nonirritating and nonsensitizing. The effects of typical penetration enhancers on corneal penetration of ophthalmic drugs are summarized in Table 2.

### *1. Surfactants*

Surfactants are used in ophthalmic solutions to improve the dispersion of suspended drugs and to increase their resorption. Surfactants increase cellular permeability primarily by disturbing the plasma membrane. The cationic surfactants, benzalkonium chloride and chlorhexidine, are used as preservatives in ophthalmic formulations, although in clinical applications cationic surfactants are highly irritating and cytotoxic. The common anionic surfactants (other than the bile salts) are sodium dodecyl sulfate and dioctyl sodium sulfosuccinate, but these are also irritants and induce reflex lacrimation. The well known ability of sodium dodecyl sulfate to disrupt lipid membranes and denature proteins suggests its possible effectiveness as an absorption enhancer.

Although most studies involving ionic detergents have noted their toxic effects, non-ionic detergents have generally proven harmless to the rabbit eye, except in a few cases in which the detergents were used in very high concentrations. Non-ionic surfactants possess a polar headgroup, generally polyoxyethylene, and a nonpolar hydrocarbon chain, and are classified by their hydrophilic–lipophilic balance. However, the hydrophilic–lipophilic balance alone could not explain the absorption-enhancing activity. Some non-ionic surfactants are used in ophthalmic formulations, but they should not contain more than 1% Tween 20 or Tween 80. Anzai et al.[140] used electron spin resonance spectroscopy to demonstrate that Triton X-100 inserts its hydrocarbon chain into the phosphatidylcholine bilayer of liposomes to fluidize it. Triton X-100 has also been shown to elicit the release of trapped glucose from egg phosphatidylcholine multilamellar and unilamellar liposomes.[141] At high concentration of non-ionic surfactant in the phosphatidylcholine bilayer, surfactant–surfactant interactions form transbilayer polar defects.

Using a slit lamp fluorophotometer, Marsh and Maurice[142] determined the fluorescence in the anterior chambers of human eyes before and 1–2 hrs after topical instillation of fluorescein solutions with various surfactants (Spans 20, 40, and 85; Tweens 20, 40, 81; Aptet 100; G 1045; Brij 35 and 58; Myrj 52 and 53). The hydrophilic–lipophilic balances of surfactants were calculated and plotted with the concentrations of fluorescence. It was found that the most active range of hydrophilic–lipophilic balance for the enhancement of the fluorescence was between 16–17 in 1% solution. Only Brij 58 was harmful at this concentration. Tween20 and Brij35 were most active and their enhancing effect increased with the increase of surfactant concentration. Saettone et al.[143] plotted the relationship between the rabbit corneal hydration produced by various penetration enhancers and their enhancing effects on the *in vitro* corneal penetration of four β-blockers. Inspection of the graph showed that two non-ionic surfactants (Brij35 and 78), although active as promoters, did not increase the corneal hydration beyond the safety level. Brij35 in particular showed a superior enhancing effect on corneal penetration of atenolol without causing corneal edema.

### 2. Bile Salts

Bile salts are synthesized from cholesterol in the liver and secreted into the duodenal lumen in the form of mixed micelles containing lecithin and cholesterol. Bile salts possess polar and nonpolar portions, and thus their amphiphilic nature is useful for solubilizing dietary fats in the lumen. The common bile salts in man are the glycin- and taurine-conjugates of the dihydroxy-bile salts deoxycholic acid and chenodeoxycholic acid, and of the trihydroxy-bile salt cholic acid. Conjugation with glycine and taurine reduces pKa and increases solubility and critical micelle concentration.[144]

Small[145] demonstrated the mechanism of membrane damage by the bile salts. At

## TABLE 2
## Summary of Ophthalmic Penetration Enhancers

| Enhancers | Concentration | Drugs | Animal | Effect | Reference |
|---|---|---|---|---|---|
| Surfactants | | | | | |
| Spans 20, 40 and 85, Tweens 20, 40 and 81, Aptet 100, G 1045, Brij 35 and 58, Myrj 52 and 53 | 1% | Fluorescein | Human | Enhanced aqueous humor concentration. Tween 20 and Brij 35 at HLB 16-17 are most effective and dose dependent | 142 |
| BL-9 | 0.1% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 3.4 times for atenolol and 7.3 times for timolol | 143 |
| Brji 35, 78 | 0.05% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 3.9-10.5 times for atenolol and 1.5-3.9 times for timolol | 143 |
| Brji 98 | 0.05% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 2 times for betaxolol | 143 |
| Bile acids | | | | | 143 |
| Deoxycholic acid | 0.05% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 1.9 times for atenolol, 5.3 times for timolol, 1.4 times for levobunolol, and 2.2 times for betaxolol | 143 |
| | 0.025-0.1% | Timolol | Rabbit | Enhanced Papp 2.5-8.3 times | 143 |
| Taurocholic acid | 1.0% | Atenolol, carteolol, tilisolol, timolol, befunolol | Rabbit | Enhanced Papp 2.4 times for atenolol, 1.5 times for carteolol, 1.4 times for tilisolol, and 2.1 times for timolol | 155 |
| | 1.0% | FD-4, FD-10 | Rabbit | Enhanced Papp 4.5 times for FD-4 and 7.1 times for FD-10 | 174 |
| | 10 mM | 6-Carboxyfluorescein | Rabbit | Enhanced penetrated amount 7.2 times | 148 |
| | 2-10 mM | FD-4 | Rabbit | Enhanced penetrated amount slightly | 148 |
| Taurodeoxy-cholic acid | 0.05% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 5.8 times for atenolol and 1.6 times for timolol | 143 |
| | 0.075-0.1% | Timolol | Rabbit | Enhanced Papp 5.2-5.5 times | 143 |
| | 10 mM | 6-Carboxyfluorescein | Rabbit | Enhanced penetrated amount 593 times | 148 |
| | 2-10 mM | FD-4 | Rabbit | Enhanced penetrated amount 30.9-61.5 times | 148 |

| Enhancers | Concentration | Drugs | Animal | Effect | Reference |
|---|---|---|---|---|---|
| Ursodeoxycholic acid | 0.05% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 2.1 times for timolol and 1.6 times for betaxolol | 143 |
| | 0.075-0.1% | Timolol | Rabbit | Enhanced Papp 8.3-11.0 times | 143 |
| Taurourso-deoxycholic acid | 0.05% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 3.0 times for atenolol and1.5 times for levobunolol | 143 |
| | 0.075-0.1% | Timolol | Rabbit | Enhanced Papp 3.3 times at 0.1% | 143 |
| Fatty acid | | | | | |
| Capric acid | 0.5% | Atenolol, carteolol, tilisolol, timolol, befunolol | Rabbit | Enhanced Papp 20.3 times for atenolol, 8.9 times for carteolol, 5.1 times for tilisolol, and 3.0 times for timolol | 155 |
| Preservatives | | | | | |
| Benzalkonium chloride | 0.01% | Prostaglandin $F_{2\alpha}$, pilocarpine, dexamethasone | Pig | Enhanced Papp 7.2 times for prostaglandin $F_{2\alpha}$, 1.7 times for pilocarpine, and 3.3 times for dexamethasone | 161 |
| | 0.01% | Tilisolol, FD-4, FD-10 | Rabbit | Enhanced Papp 3.5 times for tilisolol, 28.8 times for FD-4, and 37.1 times for FD-10 | 160 |
| | 0.02% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 5.2 times for atenolol, 2.7 times for timolol, and 1.3 times for betaxolol | 143 |
| | 0.05% | FD-4, FD-10 | Rabbit | Enhanced Papp 43.6 times for FD-4 and 60.6 times for FD-10 | 174 |
| | 0.005-0.02% | Fluorescein | Rabbit | Increased permeability 4-25 times 0.02% | 156 |
| | 0.01-0.03% | Carbachol | Rabbit | Enhanced miotic response about 20 times | 164 |
| | 0.025% | Timolol | Rabbit | Enhanced the ocular absorption about 80% and the systemic absorption about 40% | 163 |
| Chlorhexidine digluconate | 0.01% | Pilocarpine, dexamethason | Pig | Enhanced Papp 1.5 times for dexamethasone | 161 |
| | 0.0025-0.05% | Fluorescein | Rabbit, human | Enhanced permeability significantly over at 0.005% | 167 |
| Benzyl alcohol | 0.5% | Tilisolol, FD-4, FD-10 | Rabbit | Enhanced Papp 2.6 times for FD-4 and 8.1 times for FD-10 | 160 |

Continued

## TABLE 2
## Summary of Ophthalmic Penetration Enhancers (continued)

| Enhancers | Concentration | Drugs | Animal | Effect | Reference |
|---|---|---|---|---|---|
| Chlorobutanol | 0.5% | Pilocarpine, dexamethasone | Pig | Enhanced Papp 1.8 times for pilocarpine and 4.7 times for dexamethasone | 161 |
| 2-Phenylethanol | 0.5% | Tilisolol, FD-4, FD-10 | Rabbit | Enhanced Papp 2.7 times for tilisolol, 5.6 times for FD-4, and 4.8 times for FD-10 | 160 |
| Paraben | 0.04% | Tilisolol, FD-4, FD-10 | Rabbit | Enhanced Papp 1.9 times for FD-10 | 160 |
| Propyl paraben | 0.02% | Dexamethasone | Pig | Enhanced Papp 1.5 times | 161 |
| Chelating agent | | | | | |
| EDTA | 0.5% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp1.4 times for atenolol | 143 |
| | 0.5% | Atenolol, carteolol, tilisolol, timolol, befunolol | Rabbit | Enhanced Papp 1.7 times for atenolol, 2.9 times for carteolol, 2.3 times for timolol, and 1.6 times for befunolol | 155 |
| | 0.5% | FD-4, FD-10 | Rabbit | Enhanced Papp 15.5 times for FD-4 and 39.0 times for FD-10 | 174 |
| | 0.1-0.5% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 31 times for atenolol and 1.9 times for timolol at 0.5% | 47 |
| | 0.05% | Timolol | Rabbit | Enhanced ocular and systemic absorption significantly | 163 |
| Others | | | | | |
| Azone | 0.025-1.0% | Cimetidine | Rabbit | Enhanced Papp 14.1-87.0 times | 177 |
| | 0.1-0.5% | Acetazolamide, sulfacetamide, guanethidine, cimetidine, bunolol, prednisolone, flurbiprofen, flurbiprofen amide | Rabbit | Enhanced Papp 29.1 times for acetazolamide, 16.3 times for sulfacetamide, >87.3 times for guanethidine,31.3 times for cimetidine , 2.2 times for bunolol, and 2.2 times for prednisolone | 177 |

| Enhancers | Concentration | Drugs | Animal | Effect | Reference |
|---|---|---|---|---|---|
| Others - Continued | | | | | |
| | 0.025-0.1% | Cimetidine | Rabbit | Enhanced ocular bioavailability 3.9-22.0 times after instillation in rabbits | 175 |
| | 5% | Cyclosporine | Rabbit | Enhanced a penetration into the cornea and rapidly achived a steady-state of drug levels | 176 |
| Hexamethylene lauramide | 0.025-1.0% | Cimetidine | Rabbit | Enhanced Papp 17.4-64.3 times | 177 |
| Hexamethylene octanamide | 0.025-1.0% | Cimetidine | Rabbit | Enhanced Papp 5.7-100.3 times | 177 |
| Decylmethyl-sulfoxide | 0.025-1.0% | Cimetidine | Rabbit | Enhanced Papp 25-77 times | 177 |
| Saponin | 0.05% | Atenolol, timolol, levobunolol, betaxolol | Rabbit | Enhanced Papp 16.5 times for atenolol, 11.0 times for timolol, 1.3 times for levobunolol, 2.0 times for betaxolol | 143 |
| | 0.01-0.025% | Timolol | Rabbit | Enhanced Papp 2.1 times at 0.01%, 3.3 times at 0.015%, and 8.3 times at 0.025% | 143 |
| | 0.5% | Atenolol, carteolol, tilisolol, timolol, befunolol | Rabbit | Enhanced Papp 31.9 times for atenolol, 13.2 times for carteolol, 7.6 times for tilisolol, 3.3 times for timolol, 2.7 times for befunolol | 155 |
| | 0.5% | FD-4, FD-10 | Rabbit | Enhanced Papp 100 times for FD-4 and 114 times for FD-10 | 174 |

FD-4: FITC-dextran (average molecular weight 4,400), FD-10: FITC-dextran (average molecular weight 9,400).
Papp: apparent permeability coefficient, HLB: hydrophilic-lipophilic balance

low concentration, the bile salt is intercalated between the phospholipid molecules as dimers or tetramers that minimize contact of the polar hydroxyl-containing portion with the nonpolar phospholipid acyl chains. At high concentration, extensive contacts of bile salts occur in the bilayer plane, ultimately resulting in fragmentation of the bilayer and formation of mixed micelles. In other words, below the critical micelle concentration, bile salts can elicit increased bilayer permeability, but at higher concentration, bile salts cause bilayer disruption and micellar dissolution.

O'Connor et al.[146] observed that release of the entrapped marker 6-carboxyfluorescein from egg phosphatidylcholine liposomes was enhanced by cholic and ursodeoxycholic acids. Fasano et al.[147] also reported alteration in the tight junctions and increased penetration of lactulose following perfusion of chenodeoxycholic acid or ursodeoxycholic acid at low concentration (below the critical micelle concentration). Morimoto et al.[148] examined the effect of taurocholic acid and taurodeoxycholic acid on the *in vitro* rabbit corneal permeability of hydrophilic molecules and macromolecular compounds. Taurodeoxycholic acid markedly increased the corneal permeability of these penetrants at concentrations of 2 and 10 mM. Saettone et al.[143] investigated the efficacy and toxicity of a series of prospective ocular penetration enhancers (benzalkonium chloride, EDTA, non-ionic surfactants, surface-active heteroglycosides, and bile salts). The bile salts deoxycholic acid at 0.05% and taurodeoxycholic acid at 0.075% and 0.1% enhanced the permeability coefficient of timolol to a significant extent (5.3–11.0 times), without significantly increasing the corneal edema with respect to the controls.

Bile salts are endogenous residents of the gastrointestinal lumen and, as such, are thought to be nontoxic. However, bile salts are capable of permeabilizing and dissolving membranes and proteins, and can exhibit some local toxicity. A structural analog, sodium taurodihydrofusidate, has been reported to be an effective mucosal absorption enhancer.[149] Gullikson et al.[150] found that dihydroxy bile salts, but not trihydroxy bile salts, enhanced the absorption of inulin, dextran, and albumin in perfused rat jejunum. The absorption-promoting efficacy of the bile salts correlated with both the hydrophobicity and the hemolytic activity toward sheep erythrocytes. In contrast to trihydroxy bile salts, dihydroxy bile salts are more hydrophobic, more rapidly absorbed, more effective as absorption enhancers, and more damaging morphologically.

### 3. Fatty Acids

Fatty acids are very common excipients used in formulation and development of many types of dosage forms. Numerous reports have described the enhancing action of fatty acids on rectal and small intestinal absorption. Golden et al.[151] have indicated that oleic acid exerts a significant effect on the *stratum corneum* lipids by lowering their transition temperature in addition to increasing the conformational freedom or flexibility of the endogenous lipid alkyl chains above their transition temperature. Using electron spin resonance spectroscopy, Muranishi[152] demonstrated that fatty acids per-

turb the membrane structural integrity by their incorporation into the plasma membrane. Hayashi et al.[153] demonstrated the enhancing mechanism of capric acid on rectal absorption and showed that capric acid affected both the transcellular and paracellular pathways. A good relationship was observed between the enhanced penetration of several hydrophilic compounds by capric acid, and the molecular weight of the compounds. The effect of capric acid on the paracellular pathway was related to the $Ca^{2+}$-dependent contraction of the perijunctional actomyosin ring, as well as the chelation of $Ca^{2+}$ around the tight junction. Tomita et al.[154] indicated that caprylic acid interacted mainly with proteins, whereas capric acid interacted with both proteins and lipids.

Sasaki et al.[155] reported that capric acid (0.5%) showed a marked enhancing effect on the corneal and conjunctival permeability of various β-blockers, especially hydrophilic atenolol (20 times enhancement). The cornea is more susceptible than the conjunctiva to the enhancing effect of capric acid. Capric acid markedly enhanced corneal and conjunctival penetration of hydrophilic macromolecules, which are considered to penetrate via the paracellular pathway. Kato and Iwata[119,120] also reported that fatty acids significantly increased the corneal permeability of bunazosin. However, these authors strongly suggested that the contribution to the enhancing effect was due to ion pair formation rather than membrane disturbance.

### 4. Preservatives

The pharmacopoeia requires that aqueous preparations supplied in multidose containers contain antimicrobial preservatives and chelating agents at appropriate concentrations to maintain the sterility and chemical stability of the preparations. The preservatives used include benzalkonium chloride, cetylpyridium chloride, thimerosal and methyl mercury compounds, chlorobutanol, methyl paraben and propyl paraben, chlorhexidine digluconate, sodium bisulfite, and sorbic acid. The bactericidal or bacteriostatic activity of the preparation, as well as its potency as an irritant, depend on the concentration of the preservative.

Although there have been many reports on the possible negative effects of these preservatives on the integrity of the corneal epithelium and endothelium, some investigators have demonstrated the use of safe preservatives as absorption enhancers for drug delivery.[156–160] In fact, ophthalmic preservatives may be considered relatively safe as absorption enhancers for drug delivery since they have already been used in instilled droplets for clinical ophthalmic diseases over long periods. Recently, however, some ophthalmic preparations have been developed without additives in disposable dosage forms to protect the cornea from cumulative damage by preservatives.

Camber and Edman[161] investigated the influence of some commonly used preservatives on corneal permeability and the uptake of pilocarpine and dexamethasone. They found that the exposure of the isolated porcine cornea to 0.01% benzalkonium chloride or 0.5% chlorobutanol for 4 hrs almost doubled the transcorneal flux of pilo-

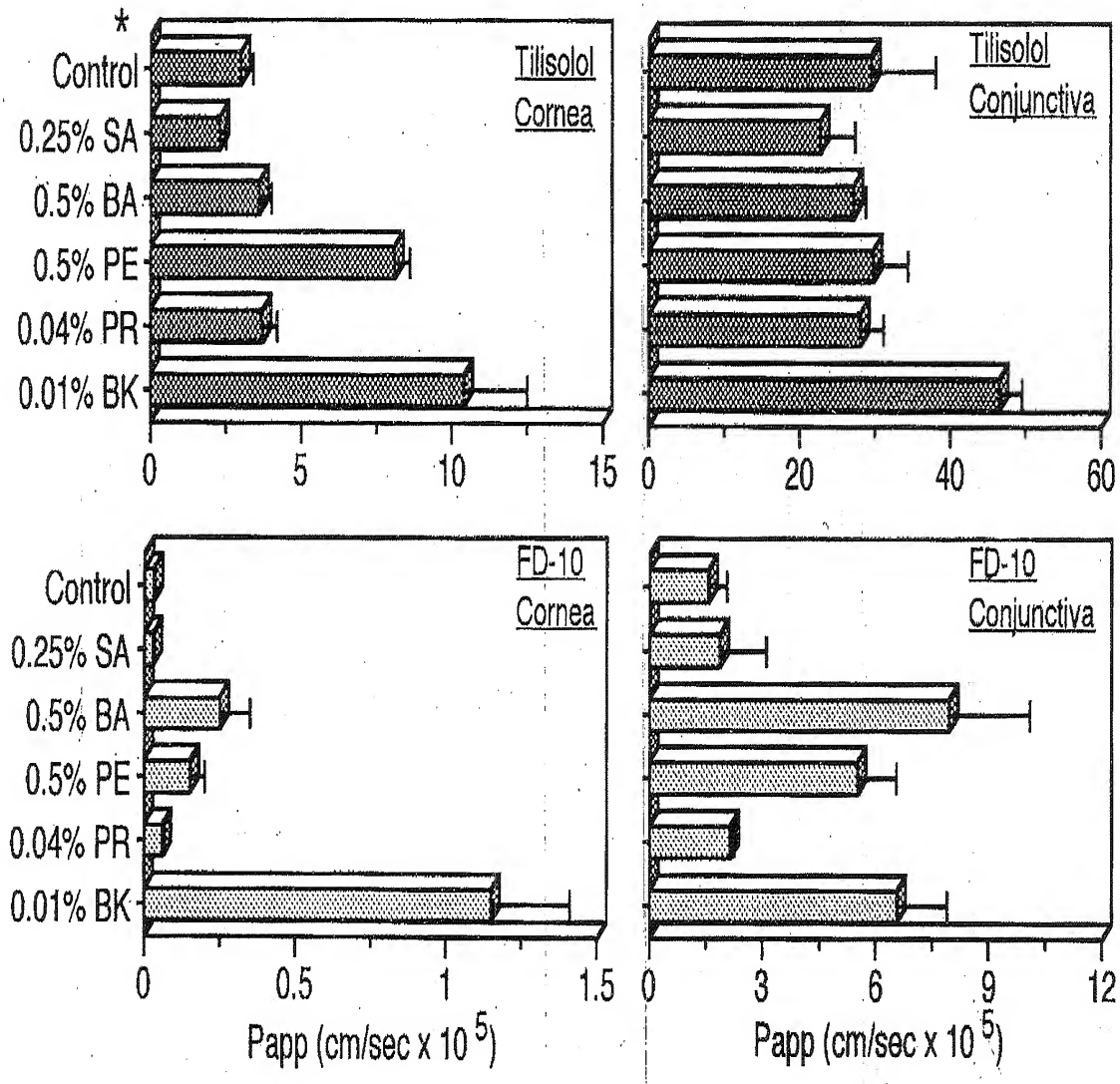



**FIGURE 13.** Effect of ophthalmic preservatives on the cornea and conjuntiva permeability coefficients (Papp) of tilisolol and FITC–dextran (mw 9400: FD–10). (SA) Sorbic acid, (BA) benzyl alcohol, (PE) 2–phenylethanol, (PR) paraben, (BK) benzalkonium chloride.

carpine, but chlorhexidine digluconate (0.01%) and a mixture of 0.04% methyl paraben and 0.02% propyl paraben were less effective. Sasaki et al.[160] also demonstrated that most typical ophthalmic preservatives significantly enhanced the corneal and conjunctival penetration of not only the β-blocker, tilisolol, but also the hydrophilic macromolecule, FITC-dextran (Figure 13). Their results suggested that ophthalmic preservatives enlarged intercellular spaces or disrupted cytoplasmic membranes in the cornea and conjunctiva superficial cells. The ophthalmic preservatives used were benzalkonium chloride, paraben, 2-phenylethanol, benzyl alcohol, and sorbic acid. Among them, benzalkonium chloride showed the highest enhancing effect. In a subsequent study, Sasaki et al.[162] found that the ophthalmic preservatives were less irritating to rabbit eyes than the absorption promoters.

Many reports on preservatives have focused on the high ocular permeability enhancing effect of benzalkonium chloride at extremely low concentration. Benzalkonium chloride is a cationic surfactant widely used as a preservative because of its bactericidal efficacy and relatively low toxicity; it tends to act on microbes indiscriminately by solubilizing cell membranes. Podder et al.[163] reported that benzalkonium chloride increased the ocular absorption of timolol in rabbits by approximately 80%, but that systemic timolol absorption was also increased and no change was observed in the absorption ratio between the eye and systemic circulation. Smolen et al.[164] demonstrated that benzalkonium chloride and another cationic surfactant, diethylaminoethyl dextran, enhanced the miotic response of topically applied carbachol. The miotic response of 0.1% carbachol in the presence of these additives was similar to that of 2% carbachol solution.

Instillation of 0.01% benzalkonium chloride shows no ocular irritation according to the Draize score, and at 0.004% to 0.01% had no influence on the epithelial aerobic metabolism.[165] Also, corneal exposure to multiple drops of benzalkonium chloride leads to epithelial accumulation but no penetration into the anterior chamber.[159] Benzalkonium chloride was also demonstrated to induce dose-dependent morphological changes in the epithelium, causing partial loss of surface microvilli. In another study, physiological changes in the epithelium occurred within 1 min and disappeared at 2 hrs after instillation of 0.02% benzalkonium chloride.[166] It is important to remember that species differences should be considered in the evaluation of preservatives. In this regard, Burstein[167] suggests that rabbits are more sensitive to single doses of preservatives than humans and found that benzalkonium chloride 0.01% increased the anterior chamber fluorescence level after instillation in rabbits but not in human subjects.

### 5. Ethylenediaminetetraacetic Acid (EDTA)

EDTA is used to stabilize ophthalmic drugs against oxidation by chelation of such divalent cations as calcium, an important ion for the regulation of cell junctions, since its chelation dilates the intercellular spaces. Therefore, EDTA is believed to loosen

the tight junctions of the corneal epithelium. However, the depletion of $Ca^{2+}$ does not act directly on the tight junctions, but rather induces global changes in the cells, including the disruption of actin filaments and adherent junctions, and the activations of protein kinases.[133] The action of cadherin, present in adherent junctions, depends on $Ca^{2+}$, and the inhibition of cadherin–cadherin interactions may loosen tight junctions. Although cadherins are not themselves present in the tight junctions, they have been strongly implicated as the major cell adhesion molecules responsible for the overall physical adhesion of cells. Yamashita et al.[168] examined the effect of an applied transmucosal potential difference on the penetration of sulphanilic acid through rat intestine and concluded that the penetration-enhancing effect of 10 mM EDTA occurred exclusively thorough paracellular pathways. EDTA also induced net water secretion into the lumen, and at 20 mM EDTA, ouabain reduced the increased flux of water and gentamicin sulfate, suggesting the involvement of active transport mechanisms.[169] EDTA has been shown by means of fluorescence and confocal microscopic technologies to act not only on cell junctions but also through disruption of the plasma membrane of rabbit corneal epithelial cells.[170] In addition, *in vivo* administration of 0.1% EDTA to normal or keratectomized corneas for 2 days was shown to induce morphological changes in stromal cells.[171]

Grass et al.[172] demonstrated the enhancing effect of EDTA on the corneal penetration of various organic compounds, especially polar compounds, and Ashton et al.[47,173] showed that EDTA increased rabbit corneal epithelial permeability to hydrophilic drugs. However, Marsh and Maurice[142] found that neither 0.34% nor 1.0% EDTA eye drops had any influence on the anterior chamber fluorescein concentration in man after topical instillation of this hydrophilic substance. EDTA also enhanced the corneal permeability of not only low-molecular-weight drugs but also of hydrophilic macromolecular drugs.[174] On the other hand, the absorption of lipophilic drugs, levobunolol and betaxolol, that penetrate the corneal epithelium via the transcellular pathway was unaffected by EDTA.[47] In experiments on rabbits, EDTA increased both ocular and systemic absorption of topically applied timolol, but the ratio of ocular absorption to systemic absorption of timolol was not affected.[163]

Topically applied EDTA itself was found to reach the iris–ciliary body in concentrations high enough to alter the permeability of the blood vessels in the uveal tract and indirectly accelerating drug removal from the aqueous humor.

### 6. Others

1–Dodecylazacycloheptan–2–one (Azone®) is a novel percutaneous penetration enhancer that effectively promotes the absorption of various drugs at low concentration without severe side effects. Ismail et al.[175] examined the cytotoxicity of Azone for ophthalmic use in rabbits. Cytotoxicity was graded according to severity and area of involvement and was then compared to the initial observations made prior

to initiation of the study. Azone at all three doses (0.7%, 0.4%, and 0.1%) in the one-day acute test was slightly discomforting to the eyes and irritating to the conjunctiva. Corneal toxicity was also observed after 16 doses of 0.7% or 0.4% Azone in one day. Newton et al.[176] dissolved cyclosporine in Azone and applied it topically to allografted rabbit eyes. Clinically significant concentrations of cyclosporine were found in the treated corneas and resulted in suppression of the incidence of graft rejection determined by clinical observation, but little or no drug was found in the aqueous humor or blood of the treated animals. Tang–Liu et al.[177] reported the effect of Azone on corneal penetration of various drugs having hydrophilicity, moderately lipophilicity, or lipophilicity. Azone at 0.1% increased the corneal penetration of hydrophilic compounds by at least 20-fold. Moderately lipophilic bunolol and prednisolone were enhanced by a factor of 2–5 by Azone at 0.025%–0.1%. However, Azone inhibited rather than enhanced the corneal penetration of the lipophilic flurbiprofen and its amide analog. These authors also compared the effects of four enhancers (Azone, hexamethylenelauramide, hexamethyleneoctanamide, and decylmethylsulfoxide) on the corneal penetration of cimetidine, and indicated that all enhancers showed a similar enhancing effect, as well as a similar effect on corneal hydration after incubation *in vitro*.

Cyclodextrins are a group of homologous cyclic oligosaccharides consisting of six, seven, and eight glucose units, namely α-, β-, and γ-cyclodextrin, respectively. Cyclodextrins are host molecules capable of forming inclusion complexes with guest molecules by absorbing the whole molecule or some part of it into their central cavity.[178] Cyclodextrins have multiple uses in the pharmaceutical, food, and cosmetic industries for increasing the solubility and stability and decreasing the toxicity of complexed molecules. The large and very hydrophilic cyclodextrin molecules cannot penetrate biological membranes but may improve the ocular bioavailability of hydrophobic drugs by solubilizing and delivering them to the surface of the cornea, where they partition into the eye. And topical instillation of 0.075% cyclosporine in α-cyclodextrin was shown to reduce an ocular inflammation of rabbits induced by intravitreal injection of bovine serum albumin.[179] Jansen et al.[180] demonstrated through the use of slit lamp biomicroscopy and scanning electron microscopy that hydrophobic cyclodextrin derivatives (dimethyl-β-cyclodextrin) were able to modify the skin barrier by extraction of components such as cholesterol and triglycerides from the biological membrane.

Saponin is a type of glycoside widely distributed in plants and consists of a sapogenin, as the aglucon moiety of the glycoside, and a sugar. Saponin is an amphiphilic compound that has surface activity. Pillion et al.[181] demonstrated that purified Quillaja saponin has the ability to promote the nasal absorption of gentamicin antibiotics in mice and rats, and nasal or ocular absorption of insulin in rats. Sasaki et al.[155,174] reported a significant enhancing effect of saponin on corneal and conjunctival penetration of β-blocker and hydrophilic macromolecules, although with severe irritation, in rabbit eyes.

TABLE 3
Summary of Ophthalmic Iontophoresis

| Location | Drug | Probe Diameter (mm) | Current x Duration (mA) (min) | Peak level (μg/ml) | Reference |
|---|---|---|---|---|---|
| Transcorneal iontophoresis | | | | Aqueous humor | |
| | Gentamicin | 10.0-20.0 | 1.0 x 1.0 | 9.0 | 186 |
| | | 3.0 | 0.2 x 10.0 | 54.8 | 182 |
| | Tobramycin | 11.0 | 0.8 x 10.0 | 312.8 | 188 |
| | Vancomycin | 3.0 | 0.5 x 5.0 | 20.2 | 189 |
| | Vidarabine monophosphate | 10.0-20.0 | 0.5 x 4.0 | 0.5 | 187 |
| | Ketoconazole | 3.0 | 1.5 x 15.0 | 1.4 | 190 |
| Transscleral iontophoresis | | | | Vitreous body | |
| | Gentamicin | 3.0 | 2.0 x 10.0 | 53.4 | 182 |
| | | 2.4 | 2.0 x 12.0 | 10.0-20.0 | 192 |
| | | 1.0 | 2.0 x 10.0 | 207.0 | 193 |
| | | 1.0 | 1.5 x 10.0 | 82.7 | 194 |
| | Ciprofloxacin | 3.0 | 5.0 x 15.0 | 0.1 | 195 |
| | Cephazolin | 1.0 | 2.0 x 10.0 | 119.0 | 193 |
| | Ticarcillin | 1.0 | 2.0 x 10.0 | 94.0 | 193 |
| | Vancomycin | 3.0 | 3.5 x 10.0 | 13.4 | 189 |
| | Foscarnet | 0.5 | 1.0 x 10.0 | 200.0 | 185 |
| | Ketoconazole | 3.0 | 5.0 x 15.0 | 0.1 | 190 |

## V. PHYSICAL ENHANCEMENT FOR THE OCULAR PENETRATION OF DRUGS

The third approach for improving the ocular permeability of drugs is to modify the integrity of the corneal epithelium and sclera, transiently, by physical means. This approach can be accomplished by exposing the eye to iontophoresis and phonophoresis. The physical approach has one major advantage over the above considered techniques in that the flux of the penetrant can be precisely controlled by varying the applied physical power, and thus the drug-delivery therapy can be more accurately tailored for the patient.

### A. Iontophoresis

Iontophoresis, a noninvasive technique for introducing ionic drugs into tissues by the means of an electric current,[182] has been used in medicine for many years. The technique was first introduced in early 1900 for transcutaneous administration of strychnine into rabbits suffering from fetal seizure, and has since been employed in ophthalmology for the treatment of corneal ulcers, keratitis, and episcleritis (Table 3). However, although physical methods can be effective for delivering ophthalmic drugs, including antibacterial, antiviral, antimetabolite, antifungal, and steroid drugs, to the

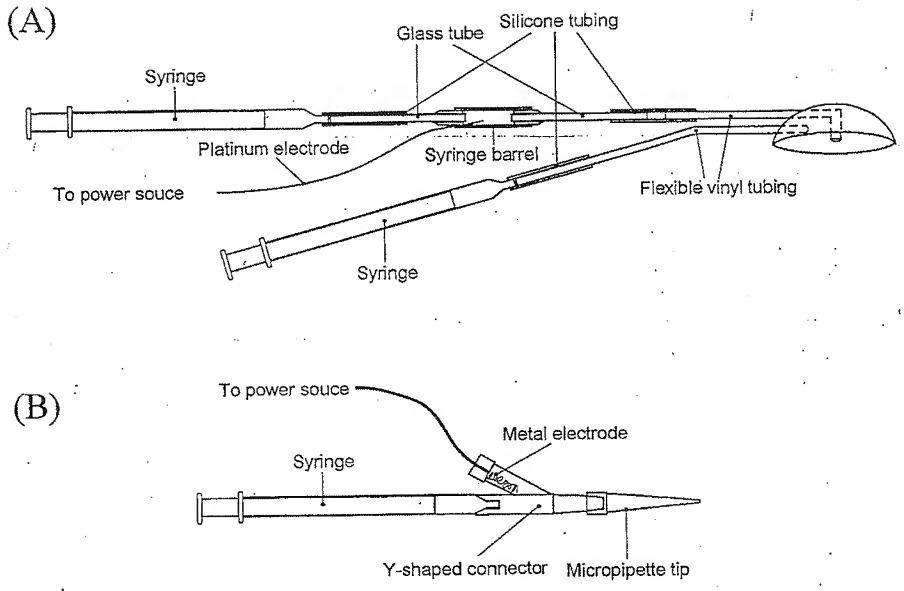



FIGURE 14. Schematic illustration of electrode and probe for ophthalmic iontophoresis. (A) Reported by Barza et al.[184] (B) Reported by Sarraf et al.[185]

cornea, aqueous humor, and vitreous body for the treatment of intraocular diseases,[183] iontophoresis has not become popular because it requires the use of inconvenient equipment and has specific electrical requirements. In iontophoresis, the drug is applied with an electrode carrying the same charge as the drug, while the ground electrode, which is of the opposite charge, is placed elsewhere on the body to complete the circuit.[183] Figure 14 shows two types of probes, each containing an electrode and a drug solution. Probe A has an eye cup, the shape of which may vary, that is held in place by the negative pressure obtained by pulling out the plunger of the syringe.[184] Probe B has a simpler design.[185] For both probes, the salt form of a drug is usually used, since the dissociated salt is highly soluble in water and has a high charge density. The advantages of iontophoresis over other drug-delivery techniques include the minimization of systemic exposure to the drug, delivery of the drug directly into the target region, and improved consistency of drug penetration.[186] However, it is also clear that iontophoresis also causes some damage to ocular tissues, although such damage may be minimized by using the lowest current densities and shortest duration of iontophoresis needed for ensuring an adequate penetration of the drug.[183]

In the field of ophthalmology, there are two types of iontophoresis, transcorneal and transscleral. Transcorneal iontophoresis can deliver and sustain high concentrations of drugs into the anterior segment of the eye, particularly into the cornea and

the aqueous humor.[182,186–190] However, little drug reaches the vitreous body via this route of administration because of the hindrance of the crystalline lens. Numerous studies have demonstrated that transcorneal iontophoresis of antibiotics offered a potentially effective method for the management of corneal ulcers. In a rabbit model of corneal ulcers, iontophoresis of tobramycin delivered significantly greater corneal concentrations of drug than did administration by topically applied drops.[191] The efficacy of treatment by iontophoresis or topical instillation of tobramycin was investigated in rabbit eyes after intrastromal injection of Pseudomonas,[188] and it was found that iontophoresis resulted in significantly fewer bacterial colonies in the cornea. And minimal surface pitting of the cornea, immediately after transcorneal iontophoresis, was reported using scanning electron microscopy.[187] On the other hand, iontophoresis can produce complications, including epithelial edema and other abnormalities, and decrease of endothelial cells.[186,188,189]

The second type of iontophoresis, transscleral iontophoresis, has been used successfully to deliver gancyclovir, foscarnet, gentamicin, cefazolin, vancomycin, and ticarcillin into the vitreous body at effective doses for treatment of such posterior segment diseases as cytomegalovirus retinitis and endophthalmitis.[182,185,189,190,192–194] The lens–iris barrier can be bypassed in transscleral iontophoresis by applying the current through the pars plana of the sclera. Transscleral iontophoresis with the negatively charged form of ciprofloxacin was shown to result in higher drug concentrations in the vitreous body than that with the positively charged form.[195] In an endophthalmitis model in rabbits, combination therapy by iontophoresis and an intravitreal injection of gentamicin resulted in significantly lower numbers of bacterial colonies than did the injection alone.[196] Therefore, transscleral iontophoresis may replace or supplement intravitreal injections for the treatment of endophthalmitis.

Successful delivery of foscarnet into the vitreous body by iontophoresis may constitute a more acceptable procedure than the long-term local therapy of cytomegalovirus retinitis in patients with AIDS.[185] However, transscleral iontophoresis may be associated with damage to the choroid and retina, as shown by Barza et al.,[193] who reported hemorrhagic necrosis, edema, and inflammatory infiltration of the retina, choroid, and ciliary body of rabbits 3 hrs after transscleral iontophoresis of gentamicin and cephazolin.

## B. Phonophoresis

The use of ultrasound, or phonophoresis, as a physical drug-penetration enhancer has also been investigated, especially in the field of transdermal drug absorption. In the 1950s, the efficacy of ultrasound was demonstrated in the field of physical medicine for treatment of localized skin conditions and delivery of drug into inflamed joints. The more recent development of innovative electronics, and the development of controlled clinical studies, should now be applied to determine whether phonophoresis is a safe and effective method for drug delivery in human patients. When an ultrasonic

pulse is sent into soft tissue, the attenuation of ultrasound occurs primarily through three processes—absorption, reflection, and scattering. The attenuation of the ultrasound depends on the wavelength of the sound, and on the density and heterogeneity of the tissue. The average attenuation of an ultrasound beam in human soft tissue is 1 dB/cm/MHz.[197,198] Ultrasound can be transformed into several other forms of energy via thermal, cavitational, or stress mechanisms.[197,198] The absorbed energy would enhance the fluidity of the barrier domains and increase the kinetic energy of the drugs, both of which would increase mass transfer across the membrane. The energy transfer may be extensive and damaging to membranes in cases where the incident frequency and intensity are large. Babizhayev et al.[112] reported ophthalmic application of phonophoresis for topical application of a prodrug of the antioxidant L-carnosine, N-acetylcarnosine. The application of therapeutic ultrasound was performed in topically anesthetized rabbits within 5 min of the exposure time to the prodrug, at an ultrasound intensity of 0.2 W/cm$^2$ and frequency of 880 kHz. Ultrasound-induced phoresis of 1% N-acetylcarnosine resulted in higher concentration of L-carnosine in aqueous humor than by either topical corneal instillation or subconjunctival injections of L-carnosine. More information is needed on the mechanism of action of these energy forms and the practical phonophoretic conditions that are tolerated by the eye. Also, other physical drug-penetration enhancements using heat or laser energy may be more effective on drug penetration through biological membranes.

## VI. CONCLUSION

Recently, various peptide drugs and oligonucleotides developed within the field of biotechnology have begun to see clinical use. However, the best therapeutic candidates among these drugs are characterized by poor ocular bioavailability due to their impermeability and instability. Therefore, at this time the treatment of posterior segment disorders is still limited to conventional forms of drug administration. Of interest also is that the ocular route may be useful for systemic delivery of conventional and peptide drugs because the mucous membrane in the conjunctiva and nasal cavity are also permeable to both kinds of drugs. Although the enhancement of drug ocular penetration may be very useful and may even be necessary in order to gain superior therapeutic efficacy, none of the enhancement approaches are panaceas, and they should be introduced to clinical use only after considering the balance of risks and benefits. Further information is needed to determine the nature of this balance—information on drug-penetration-enhancing mechanisms, ocular metabolism, adequate conditions for enhancing drug absorption, side effects, and the influence of ocular diseases on the specific drug-absorption-enhancement technique.

Finally, new approaches have recently been devised for brain targeting of drugs across the blood-brain barrier. These include transport via receptor endocytosis, active transport, and intercellular opening by hypertonic solution. These techniques for enhanc-

ing drug penetration might also be useful for delivering ophthalmic drugs into the eye through the blood–retinal and blood–aqueous barriers after systemic administration. Further optimization of drug delivery and resultant improvements in drug efficacy may be achieved through the rational integrated application of available pharmaceutical, chemical, and physical techniques.

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# Exhibit C

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# Tumor physiology and drug resistance

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*Key words:* microenvironment, drug resistance, tissue penetration, repopulation

## Abstract

Clinical resistance is usually assumed to be due to the initial presence or selection of drug-resistant cells in tumors. While important, it is suggested in this review that genetically-determined causes of cellular resistance represent but one cause (and possibly not the major cause) of effective clinical resistance of solid tumors. Factors that depend on tumor physiology, and on the microenvironment and three-dimensional structure of solid tumors, may have a profound influence on their sensitivity to anti-cancer drugs. Particular emphasis is placed on the limited penetration of some drugs from tumor blood vessels and on the repopulation of tumor cells between courses of chemotherapy as causes of clinical resistance. Both of these mechanisms are amenable to modulation to improve therapeutic index. Failure to recognize that clinical drug resistance cannot be explained entirely by mechanisms operative at the level of the single cell may lead to disappointing results in clinical trials such as, for example, clinical failure of the strategy of reversal of multidrug resistance.

## Introduction

Chemotherapy is of limited effectiveness for the treatment of common metastatic solid tumors of adults. While shrinkage of disease (remission) may occur in a proportion of patients with cancers of the lung, breast, colon or other sites, this is usually of limited extent and of short duration. It is rare that chemotherapy leads to disappearance of all clinically evident disease (i.e. to complete remission), and extremely rare that it leads to cure. Most solid tumors are either resistant to drugs at initiation of chemotherapy, or become resistant a few months after starting treatment.

Most of the published literature relevant to the causes of limited effectiveness of chemotherapy for human cancer has focussed on molecular mechanisms that cause individual tumor cells to become resistant to anti-cancer drugs. There is widespread acceptance of the hypothesis that tumor cells are undergoing mutation, and that drug resistance of human tumors arises because of the selection of mutant drug-resistant cells during tumor progression, or following exposure to chemotherapy. Genetically-determined causes of drug resistance include mutation or amplification of genes encoding drug export pumps such as P-glycoprotein and multidrug-resistance protein (MRP), or those leading to reduced levels of topoisomerase II, increased DNA repair, reduced ability to undergo apoptosis and others. Cells expressing these or other stable mutations have been isolated from some human tumors, so there is some evidence for the selection of genetically stable drug-resistant cells. However, at least two types of evidence suggest that such a selection might be less common than is generally accepted:

1. The selection of genetically stable drug-resistant mutant cells in experimental systems has been remarkably difficult, and often requires much higher selection pressures (prolonged exposure to mutagens, higher doses and longer duration of exposure to anti-cancer drugs) than occurs during the treatment of human tumors.
2. When human tumors relapse after initial chemotherapy, often given in the adjuvant setting, they may respond later to the same chemotherapy with a probability of response that is in the same range as would be expected for untreated tumors [1]. Thus, it seems unlikely that relapse occurred because of the selection of stable drug-resistant cells.

Molecular changes that lead to transient drug resistance may also occur at a cellular level, such as alterations in the expression of genes due to changes

in methylation [2]. Such causes of drug resistance are probably relevant to the resistance of human cancer, but because such cellular changes are unstable, they are difficult to study.

In contrast to changes in drug sensitivity that operate at the level of the individual cancer cell, drug resistance that depends on the societal nature of cells in a tumor has been studied rather rarely. In this article, I present the argument that the effective resistance of human tumors may depend frequently on tumor physiology. Specific hypotheses to be addressed are the following:

1. The microenvironment of solid tumors may have a profound effect on their sensitivity to drugs.
2. Modification of the microenvironment represents a feasible method for improving the effectiveness of chemotherapy.

*Microenvironmental factors* that may influence the sensitivity of tumors to anti-cancer drugs include the following:

1. Decreasing rate of cell proliferation at increasing distance from tumor blood vessels.
2. The presence of hypoxia and low extracellular pH.
3. Cellular contact and high cell concentration.
4. Effects of drugs on tumor blood vessels.
5. Variation in vascular density and blood flow leading to decreased and varying delivery of drugs to the tumor (as compared to normal tissues).
6. The requirement for drugs to penetrate tumor tissue from blood vessels to reach the target cancer cells.
7. Proliferation of surviving cells between courses of chemotherapy (known as repopulation).

Evidence relating to the first five of the above mechanisms is reviewed briefly below, but the focus of this article is on drug penetration through tissue and on repopulation.

## Cell proliferation

It has long been known that the rate of cellular proliferation is heterogeneous within solid tumors and decreases with increasing distance from tumor blood vessels [3–5]. Almost all anti-cancer drugs are more active against proliferating cells, so that poorly-nourished slowly proliferating tumor cells will tend to be spared.

## Hypoxia and low extracellular pH

Regions of hypoxia are known to exist in solid tumors, and may occur either because of limited diffusion from functional blood vessels (diffusion-limited or chronic hypoxia) or because of interruptions in blood flow (transient hypoxia). The effect of hypoxia to cause resistance to radiation is well known. Hypoxia may have a direct influence on the sensitivity of cultured cells to some anti-cancer drugs, although this effect is usually less than the approximately three-fold difference in sensitivity to radiation [6,7]. However, its major influence on the responsiveness of tumors to chemotherapy is probably indirect. Sustained hypoxia, together with low concentrations of other nutrient metabolites in hypoxic regions, leads to inhibition of cell proliferation (probably by depriving cells of metabolic energy). Thus, cells in chronically hypoxic regions are likely to be resistant to most anti-cancer drugs. Fluctuations in blood flow that lead to transient hypoxia also lead to interruptions in the delivery of drugs through the same blood vessels. The effect on overall tumor sensitivity is then likely to depend on the duration of interruptions in blood flow relative to the duration for which effective concentrations of the anti-cancer drug are maintained in the tumor blood vessels.

The mean extracellular pH is also lower in tumors than in normal tissues. Low extracellular pH has the effect of decreasing the ionization of weak acids and increasing the ionization of weak bases. Diffusion of drugs across the cell membrane usually occurs in the uncharged form, so that low extracellular pH leads to increased cellular uptake and activity of weak acids (such as melphalan) and decreased cellular uptake and activity of weak bases (such as doxorubicin) [8,9]. However, low pH may also lead to changes in the activity of transport proteins so that it may have less predictable effects on the cellular uptake of drugs that are actively transported into cells; for example, the activity of the weak acid methotrexate is inhibited at low pH [10]. In addition to direct effects on cellular uptake of drugs, low pH is also found in tumors at increasing distance from tumor blood vessels [11], probably because of failure to clear acidic products of metabolism, such as carbonic and lactic acid. Low extracellular pH is an additional cause of reduced proliferation of tumor cells (and hence of reduced sensitivity to chemotherapy) in these regions.

## Cellular contact and high cell concentration

At least two groups have shown that cells which are drug-sensitive in dispersed cell culture may be resistant when grown in contact as multicellular tumor spheroids or as solid tumors in mice [12,13]. This effect

is additional to that of tissue penetration described below; the mechanisms remain largely unknown.

Many drugs are tested against tumor cells in dilute tissue culture (~$10^5$ cells/ml) and there is an implicit assumption that relative cell kill will be similar at higher cell concentrations such as those that are found in solid tumors ($10^8$–$10^9$ cells/ml). This assumption of first-order kinetics is usually correct for drugs that are present in much higher concentration than their molecular targets, as is probably the case for most anti-cancer drugs that act on DNA. This assumption may not be correct for agents that must inactivate a very large number of cellular targets in order to be effective. We have shown, for example, that agents such as verapamil or cyclosporin A which reverse multiple drug resistance due to P-glycoprotein in dilute tissue culture, lose their effect as the cell concentration in tissue culture increases (Figure 1) [14]. One can therefore predict the lack of effectiveness of these inhibitors of P-glycoprotein at cell concentrations that are observed in solid tumors. Indeed, there has been failure to demonstrate effectiveness of the strategy of drug resistance reversal against established solid tumors in animals, or in randomized clinical trials.

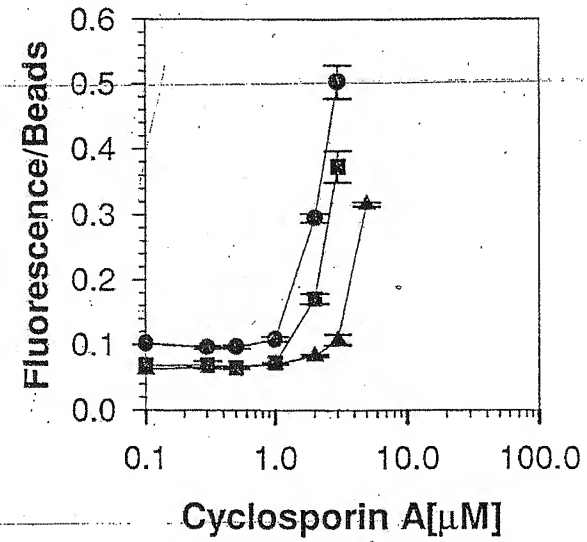


*Figure 1.* The effect of cell concentration on the ability of cyclosporin A to stimulate the uptake of doxorubicin into drug-resistant $CH^RC5$ cells which express the mdr phenotype. (Symbols: circles $10^5$ cells/ml, squares $10^6$ cells/ml, triangles $10^7$ cells/ml). Similar results were obtained using verapamil. Reproduced from Tunggal et al. [14] with permission. Note decreasing effectiveness of the reversing agent at increasing cellular concentration

[15–17]. Inactivity at high cell concentration could be a problem for some other agents which are directed against targets on the cell surface, such as those that inhibit some growth factor receptors, if there are a large number of targets per cell.

## Effects on tumor blood vessels

Inhibition of angiogenesis is 'in vogue'. Conventional anti-cancer drugs might cause secondary effects on tumor cells by inhibiting their blood supply, although there is limited evidence for such an effect, at least when large intermittent doses of chemotherapy are given. The endothelial cells lining established blood vessels in tumors are probably relatively resistant to most types of chemotherapy, because their rate of proliferation is quite slow [18,19]. The formation of new vessels by proliferating endothelial cells might be interrupted transiently, but is unlikely to lead to substantial effects against established tumors.

There are recent reports that low dose chemotherapy given frequently or continuously together with inhibitors of angiogenesis is effective against some tumors in experimental animals, perhaps because of combined effects on the cells which line established tumor blood vessels [20,21]. Clinical trials are in progress to evaluate this strategy in the treatment of human cancer.

## Poor drug delivery to tumors

The blood supply to tumors is often poor as compared to that in normal tissues. This is due to larger intercapillary distances and to variable blood flow, including stasis, in tumors as compared to normal tissues [22,23]. Since anti-cancer drugs are delivered to tumors via their blood supply, this puts them at a disadvantage as compared to normal tissues.

## Penetration of tumor tissue

Until recently, studies of drug penetration through tumor tissue depended on two methods that are indirect and technically complex:

1. Multicellular tumor spheroids can be exposed to fluorescent or radiolabeled drugs, and fluorescence or radiolabel can be related to radial penetration in histological sections or autoradiographs. This method

has been used to demonstrate limited penetration of several anti-cancer drugs including doxorubicin, methotrexate and vinblastine [24–26]. Recent refinements using confocal microscopy instead of tissue sections have confirmed limited distribution of doxorubicin in larger spheroids [27], and computerized laser scanning microsocopy has been used to demonstrate large gradients of doxorubicin concentration in human breast cancer [28].

2. The vital fluorescent dye, Hoechst 33342, has been used to establish a gradient into tissue from the periphery of spheroids, or from tumor blood vessels. Following treatment with an anti-cancer drug, the tissue is dissociated, cells are separated on the basis of Hoechst fluorescence by flow cytometry and cell sorting, and clonogenic cell survival is estimated as a function of distance into tissue. This method has confirmed that drug delivery is a major limitation for doxorubicin, although not for 5-FU and several alkylating agents [29–31]. Although the same factors that lead to slow penetration after acute administration were shown to lead to longer retention after chronic exposure [32], most of the administered drug is likely to be excreted before tissue penetration has occurred.

More recently, a conceptually simple technique has been established by Wilson and his colleagues which allows direct assessment of tissue penetration by anti-cancer drugs [33,34]. Tumor cells are grown on collagen-coated microporous teflon membranes as a multicellular layer (MCL) that has similar characteristics to tumor tissue *in vivo*. MCL typically achieve a thickness of ~200 μm, similar to the maximum distance between blood vessels and necrosis in human tumors. We have generated MCL from a variety of human and murine tumor cell lines and have shown that the MCL develop an extracellular matrix (ECM) using both non-specific staining with Masson's trichrome and by immunohistochemistry to several components of the ECM. In general, the ECM seems similar in MCL and in tumors grown from the same cell line in mice, either using syngeneic animals or nude mice to generate xenografts. We have also observed rare tight junctions between the epithelial cells in such tumors and in the corresponding MCL. Thus, MCL seems to provide a good model for studying drug penetration through solid tumor tissue that is likely to reflect drug penetration properties *in vivo*.

We and others have used MCL to study the penetration of drugs through tissue [10,35–38] There

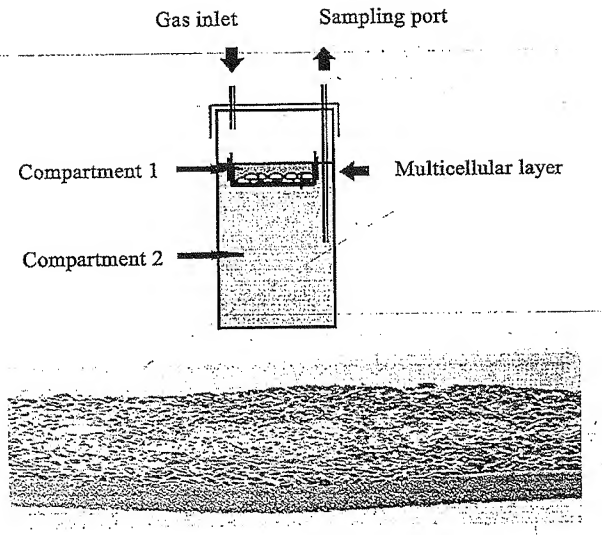


*Figure 2.* Schematic diagram of the MCL method used for assessment of drug penetration. An MCL grown on a collagen-coated teflon membrane is shown at the bottom. The MCL and its support are floated on medium in a larger vessel (top). A drug is added to compartment 1 in dilute agar and sampled as a function of time in the stirred compartment 2, on the other side of the MCL.

are differences in the experimental system used in different laboratories, but the essential features are that the drug of interest is added to one side of the MCL and is sampled from a second compartment on the other side of the MCL. The system used in my laboratory is that developed by Wilson and colleagues and is shown schematically in Figure 2. The upper compartment (compartment 1 in Figure 2) is of low volume and the drug (usually radiolabeled) is added in dilute agar to prevent convection. The time-dependent penetration of the drug into a stirred-medium-containing compartment on the other side of the MCL (compartment 2 in Figure 2) is then determined. The simplest experiment uses $^{3}H$ or $^{14}C$-labeled drugs, although the label measured in the receiving compartment may be associated with drug metabolites and hence represents an upper estimate of penetration of the parent drug. Concurrent controls evaluate drug penetration through the coated teflon membrane alone, and sucrose bearing the alternative radiolabel is used as a standard to ensure uniformity among experiments. These studies are complemented by measuring the concentration of the drug and its metabolites by analytical methods such as HPLC.

Kyle and Minchinton [37] have developed a system where the drug is added to stirred medium on one side of the MCL (which is mounted vertically between two

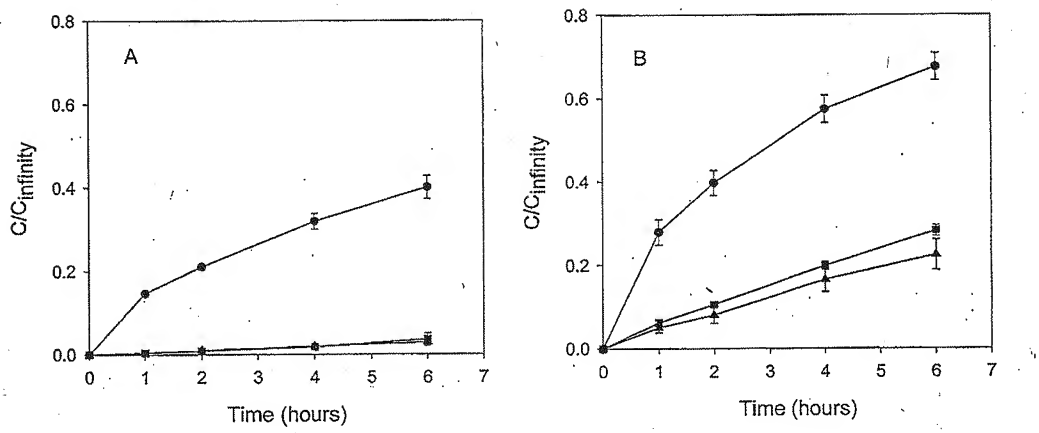


*Figure 3.* The time-dependent penetration of (A) [$^{14}$C]doxorubicin and (B) [$^{3}$H]5-fluorouracil, through MCL derived from human MCF-7 cells or murine EMT-6 cells. The concentration in compartment 2 of Figure 2 is expressed as a ratio of the expected equilibrium concentration. The upper curves represent drug penetration through the teflon membrane in the absence of an MCL. Reproduced from Tunggal et al., with permission [36]. Note very poor tissue penetration, especially of doxorubicin

chambers) and is sampled from the compartment on the other side; this has the advantage of allowing simultaneous estimation of drug concentration on both sides of the MCL, but is technically more difficult to establish.

Representative curves describing penetration of doxorubicin and 5-fluorouracil are shown in Figure 3. The penetration of all drugs that have been evaluated through MCL is slow compared to that through the teflon membrane alone and extremely poor for doxorubicin and mitoxantrone [36]. The slow establishment of equilibrium conditions in MCL is likely to reflect the situation in solid tumors, where intermittent injections of relatively high doses of drugs is followed by a peak and rapid fall in serum concentration. Cells distal from blood vessels are then likely to experience only low concentrations of most drugs in their microenvironment, and this is likely to be particularly the case for doxorubicin and mitoxantrone. When solid tumors respond to these agents, most likely they do so by repeated loss of cells close to blood vessels at the time of administration of successive courses of treatment, analogous to skinning an onion, or rather a series of inside-out onions.

It appears that the penetration of drugs through tissue is mainly through the ECM. The penetration of methotrexate through MCL, for example, is increased in the presence of acidic conditions or of folic acid, both of which inhibit the uptake of methotrexate into cells (Figure 4) [10]. Also, we have shown that penetration of doxorubicin through MCL composed of cells which express the drug export pump P-glycoprotein is enhanced compared with cells of similar origin that do not express P-glycoprotein. Moreover, agents that inhibit the function of P-glycoprotein such as verapamil, cause a decrease in tissue penetration of doxorubicin (Figure 5) [38]. This observation offers a second explanation as to the failure of strategies to reverse multiple drug resistance to show therapeutic benefit for established solid tumors, as compared to single cells in culture.

Obtaining information about the factors which lead to limited penetration of anti-cancer drugs through tissue is important, but even more important is the potential to improve tissue penetration. Since vascular access is far more limited in tumors than in normal tissues [22,23] strategies that improve tissue penetration are likely to have a much greater effect on anti-tumor effects than on toxicity to normal tissues, and may be expected to improve the therapeutic index. Potential strategies that are under investigation include modification of the ECM and inhibition of the sequestration of basic anti-cancer drugs (such as doxorubicin and mitoxantrone) in acidic compartments of cells [34,39].

## Repopulation

The proliferation of surviving tumor cells between daily doses of fractionated radiotherapy is an important cause of failure to achieve local tumor control [40–42]. There is evidence from several experimental systems that this process can accelerate with time, presumably

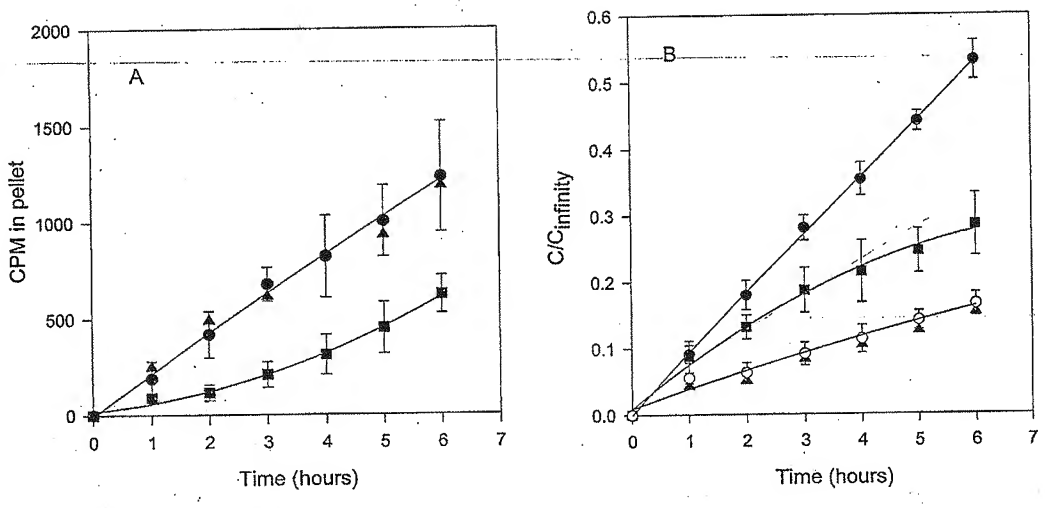


*Figure 4.* (A) The effect of acidic pH to reduce uptake of radiolabeled methotrexate into EMT6 cells in suspension (symbols: circles pH 7.3, squares pH 6.0, triangles pH 8.0). (B) The effect of acid pH to increase the time-dependent penetration of radiolabeled methotrexate through MCL derived from EMT6 cells (symbols: solid circles cell-free control, open circles pH 7.3, squares pH 6.0, triangles pH 8.0). Adapted from Cowan et al. [10] with permission. Note that poorer uptake of the drug into cells correlates with improved penetration through tissue

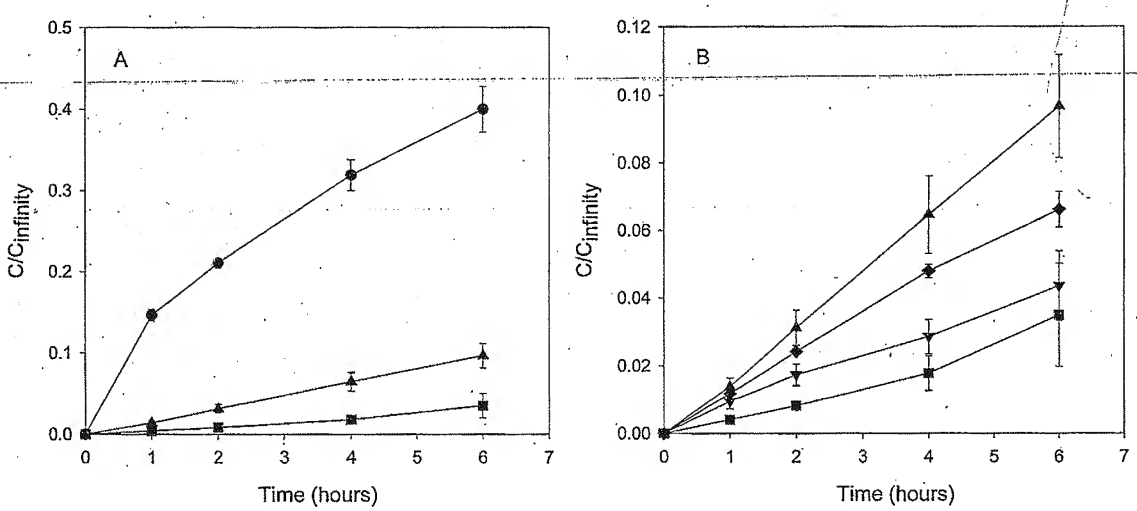


*Figure 5.* (A) Penetration of radiolabeled doxorubicin as a function of time through MCL derived from wild-type EMT6 cells (squares) and multiple drug-resistant EMT6 cells (triangles). Circles indicate penetration through the cell-free teflon membrane. (B) Penetration through MCL derived from wild-type cells (squares), mdr–expressing cells (upward triangles), and mdr-expressing cells in the presence of the reversing agents verapamil (diamonds) or GG918 (downward triangles). Data reproduced from Tunggal et al. [38] with permission. Note better penetration through MCL derived from drug-resistant cells and poorer tissue penetration with application of reversing agents

as surviving cells are stimulated to divide by improving nutrition or by the action of growth factors. Studies of the relationship between the probability of tumor control and duration of fractionated radiotherapy suggest a similar acceleration of repopulation in human tumors undergoing treatment [40,42–44]. Clinical trials using accelerated fractionation, such as continuous hyperfractionated accelerated radiation therapy (CHART) have been designed to overcome this effect and have shown therapeutic benefit [45].

Radiotherapy is most often delivered as daily dose fractions. The process of repopulation is likely to

*Table 1.* Evidence for repopulation after chemotherapy in experimental systems

| Tumor [reference] | Drug | Doubling time (untreated) | Minimum doubling time during repopulation | Delay to start of repopulation |
|---|---|---|---|---|
| 9L tumor [47] | BCNU | 1.7 days | 0.6 days (dose-dependent) | 1–4 days |
| B16 melanoma [48] | Cyclophosphamide | 2.8 days | 1.5 days | 5 days |
| | CCNU | | 0.85 days | none |
| SA-NH tumor [49] | Cyclophosphamide | | ~0.5 × doubling time of untreated tumor | 14 days |

Adapted from Davis and Tannock [46], where methods for estimating the rate of repopulation are described.
BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU = 1-(2-chloroethyl)-3 cyclohexyl-1-nitrosourea.

be more important between courses of chemotherapy which are given typically at 3-week intervals, but appears to have been rather neglected in the literature [46]. We were able to locate only three studies in which the rate of repopulation of tumor cells had been studied following the treatment of experimental tumors, while another group of investigators had studied the process following the treatment of spheroids [47–50]. The results of the *in vivo* studies are summarized in Table 1. Although the onset and rate of repopulation depended on the drug and the tumors or spheroids investigated, each of these studies found evidence for a higher rate of proliferation at some time after treatment of tumors by chemotherapy (i.e. of repopulation) than in untreated control tumors. Thus, there appears to be evidence of accelerated repopulation in experimental tumors following chemotherapy as well as during fractionated radiotherapy. The process of tumor 'recovery' may therefore be analogous to repopulation of the bone marrow from stem cells that are stimulated to divide as a result of treatment.

There are few relevant data on the rate of repopulation of human tumors following chemotherapy. It is not easy to study the proliferation of cells following tumor treatment, because at early intervals it is difficult or impossible to distinguish true surviving cells from lethally-damaged cells that have yet to undergo the morphological changes that will precede their ultimate lysis; however, this should be less of a problem at longer intervals after drug treatment. In one study, tumor cell proliferation was assessed in patients with oropharyngeal cancer following induction chemotherapy [51]; there was a higher rate of cell production in treated patients than in those that had not received chemotherapy, again consistent with accelerated repopulation. Norton and Simon [52] used Gompertzian growth curves to model tumor shrinkage and regrowth of human tumors, and suggested the possibility of increased tumor cell proliferation following shrinkage of human tumors if the relationship between tumor size and growth rate remained Gompertzian (i.e. sigmoid). However, repopulation during fractionated radiotherapy can take place in the absence of major changes in gross tumor volume, which reflects poorly changes at a cellular level.

Figure 6 provides a model for the effects of repopulation following chemotherapy [46]. In Figure 6A, where there is assumed to be no change in the rate of repopulation, a human tumor may show net growth because of this process, even if each course of chemotherapy leads to killing of 70% of the viable tumor cells. (This may be quite difficult for drugs that have limited penetration of tissue.) Figure 6B provides a model for the effects of an increase in repopulation following successive cycles of chemotherapy, such that the doubling time of surviving tumor cells decreases from 2 months (a typical volume doubling time for untreated human tumors) to about 1 week (still slower than estimated for human head and neck cancers towards the end of a period of radiotherapy) [40]. This modeling illustrates that a changing rate of repopulation can cause initial tumor shrinkage followed by regrowth, as is observed commonly in the treatment of 'sensitve' human tumors, in the absence of any change in the intrinsic drug sensitivity of the constituent cells. The process may be more complex than that illustrated by this simple modeling, because in addition to killing tumor cells, anti-cancer drugs may inhibit proliferation of surviving cells following their administration. However, this cytostatic effect is unlikely to last through the typical 3-week interval between cycles of treatment with chemotherapy. Including temporary cytostatic effects in the model does not substantially change the effect of accelerating repopulation

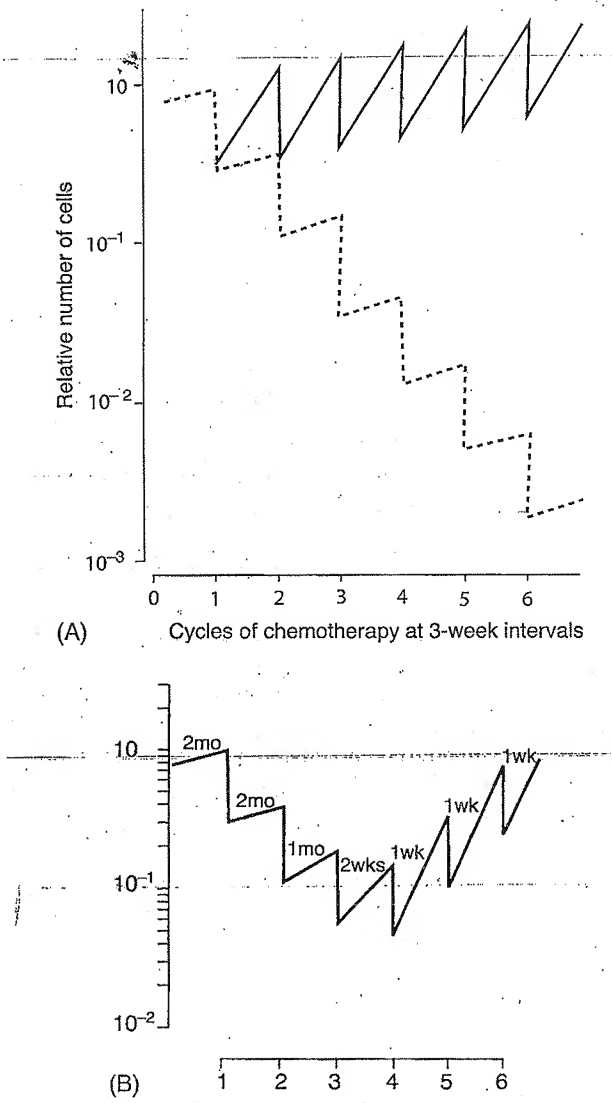


*Figure 6.* Models of cell killing and repopulation during chemotherapy. In (A), it is assumed that each 3-weekly course of treatment kills 70% of the tumor cells and repopulation is shown with a doubling time of 10 days (solid line) or 2 months (dashed line). In (B), the rate of repopulation increases between successive cycles of chemotherapy so that doubling time decreases from 2 months to 1 week. Adapted from Davis and Tannock [46] with permission. Note that shrinkage and regrowth of tumors, observed commonly in the clinic, may occur due to accelerating repopulation and without any selection of drug-resistant cells

to cause tumor shrinkage and regrowth as shown in Figure 6B [46].

As for radiotherapy, there are strategies that might be used to inhibit repopulation between courses of chemotherapy, and thereby to avoid the effective drug resistance that is observed. One method is to change the 'fractionation' and to give lower doses of drugs more frequently or continuously. However, this is unlikely to be tumor-specific, and may also lead to inhibition of tumor cell proliferation at the expense of less killing of tumor cells. Other approaches that are under investigation include the use of hormonal agents to block proliferation between cycles of chemotherapy for hormone-sensitive tumors, or inhibition of receptors to growth factors that stimulate selectively tumor cell proliferation. Such agents will need to be short acting and discontinued just before the next cycle of chemotherapy, since anti-cancer drugs are likely to be more effective in killing cycling tumor cells.

## Conclusions

It will be evident from this brief review that understanding the effects of drugs on tumors cannot be explained simply by the effects of drugs on single cells in dilute tissue culture. While genetically-based causes of drug resistance are important, they offer but partial insight into the responsiveness, or lack of it, in solid human tumors. Heterogeneity of the microenvironment and of cellular proliferation, effects due to cell contact and to high cell concentration, and the variable and imperfect vasculature in tumors are all important factors in determining drug effects. Strategies to improve the penetration of drugs through tumor tissue, and to inhibit selectively the repopulation of tumor cells offer feasible methods for improving therapeutic index, but as yet have been subject to minimal experimentation. Failure to appreciate the effect of some of the above factors can lead to costly mistakes, such as the unproductive clinical attempts to reverse P-glycoprotein by using inhibitors that were based largely on results from using single cells in culture.

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# Exhibit D

*Cancer and Metastasis Reviews* **9**: 253–266, 1990.


# Vascular and interstitial barriers to delivery of therapeutic agents in tumors

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*Key words:* vascular permeability, interstitial pressure, microvascular pressure, monoclonal antibodies, LAK cells, TILs, biological response modifiers, anti-angiogenic therapy

**Abstract**

The efficacy in cancer treatment of novel therapeutic agents such as monoclonal antibodies, cytokines and effector cells has been limited by their inability to reach their target *in vivo* in adequate quantities. Molecular and cellular biology of neoplastic cells alone has failed to explain the nonuniform uptake of these agents. This is not surprising since a solid tumor *in vivo* is not just a collection of cancer cells. In fact, it consists of two extracellular compartments: vascular and interstitial. Since no blood-borne molecule or cell can reach cancer cells without passing through these compartments, the vascular and interstitial physiology of tumors has received considerable attention in recent years. Three physiological factors responsible for the poor localization of macromolecules in tumors have been identified: (i) heterogeneous blood supply, (ii) elevated interstitial pressure, and (iii) large transport distances in the interstitium. The first factor limits the delivery of blood-borne agents to well-perfused regions of a tumor; the second factor reduces extravasation of fluid and macromolecules in the high interstitial pressure regions and also leads to an experimentally verifiable, radially outward convection in the tumor periphery which opposes the inward diffusion; and the third factor increases the time required for slowly moving macromolecules to reach distal regions of a tumor. Binding of the molecule to an antigen further lowers the effective diffusion rate by reducing the amount of mobile molecule. Although the effector cells are capable of active migration, peculiarities of the tumor vasculature and interstitium may be also responsible for poor delivery of lymphokine activated killer cells and tumor infiltrating lymphocytes in solid tumors. Due to micro- and macroscopic heterogeneities in tumors, the relative magnitude of each of these physiological barriers would vary from one location to another and from one day to the next in the same tumor, and from one tumor to another. If the genetically engineered macromolecules and effector cells, as well as low molecular weight cytotoxic agents, are to fulfill their clinical promise, strategies must be developed to overcome or exploit these barriers. Some of these strategies are discussed, and situations wherein these barriers may not be a problem are outlined. Finally, some therapies where the tumor vasculature or the interstitium may be a target are pointed out.

## Introduction

The advent of hybridoma technology and genetic engineering has led to the design and large-scale production of monoclonal antibodies (MAbs) and other biological macromolecules potentially useful for cancer detection and treatment. These molecules can be conjugated to radionuclides, chemotherapeutic agents, toxins, growth factors, enzymes, effector cells or liposomes. Moreover, a number of genetically engineered cytolytic macromolecules (e.g., cytokines) and killer cells are under active investigation as potential therapeutic agents. While the concept of using antibodies, cy-

tokines or effector cells with a high degree of specificity for cancer cells remains attractive for cancer therapy, clinical results have not, to date, lived up to the earlier promises of their perceived potential. Similarly, many of the conventional drugs of lower molecular-weight, although effective against hematologic cancers (e.g., leukemias, lymphomas), have had minimal impact on solid tumors (e.g., breast, lung, colon, brain). A key problem with blood-borne therapeutic agents is their inability to reach all regions of a tumor in adequate quantities [1]. Cellular factors (e.g., heterogeneity of tumor-associated antigen, multidrug resistance) *alone* can not account for the poor delivery of these agents in tumors. This is not surprising since a solid tumor is a pathophysiological entity, which is more than the sum of its component cells. This tissue is capable of behaving as differently from its component cells, much as the liver does when compared with hepatocytes [2]. The neoplastic cells *in vivo* are, in fact, part of an organized structure which has at least two extracellular compartments: the vasculature and the interstitium [2]. While much of cancer biology has advanced rapidly in recent years, the study of tumor pathophysiology of cancer has lagged behind.

A blood-borne molecule or cell that enters the tumor vasculature reaches cancer cells via: (a) distribution through the vascular compartment; (b) transport across the microvascular wall; and (c) transport through the interstitial compartment. For a molecule of given charge, size, and configuration, each of these transport processes may involve convection (i.e., solute movement associated with bulk solvent movement) and diffusion (i.e., solute movement resulting from solute concentration gradients). In addition, during this journey the molecule may bind non-specifically to proteins or other tissue components; bind specifically to the target(s) and/or be metabolized [3, 4]. Although LAK or TIL cells are capable of active migration, they encounter the same barriers against their movement in tumors. In this article, I will critically review these physiological barriers to delivery of molecules and cells in tumors and discuss some strategies to overcome or exploit them for therapeutic benefit.

## Distribution through vascular space

The tumor vasculature consists of (a) vessels recruited from the preexisting network of the host vasculature and (b) vessels resulting from the angiogenic response of host vessels to cancer cells [5, 6]. Movement of molecules through the vasculature is governed by the vascular morphology (i.e., the number, length, diameter and geometrical arrangement of various blood vessels) and the blood flow rate.

### *Vascular morphology*

Although the tumor vasculature originates from the host vasculature, its organization may be completely different depending upon the tumor type, its growth rate, and its location. The architecture is different not only among various tumor types, but also between a spontaneous tumor and its transplants [7].

Macroscopically, the tumor vasculature can be studied in terms of two idealized categories: peripheral and central. In tumors with peripheral vascularization, the centers are usually poorly perfused (Fig. 1). In those with central vascularization, one would expect the opposite. Hence, the penetration of blood-borne substances should follow the same pattern. In reality, a tumor may consist of many territories, each exhibiting one or the other of these two types of idealized vascular patterns.

Microscopically, the tumor vasculature is highly heterogeneous and does not conform to the standard normal vascular organization (i.e., artery to arteriole to capillaries to postcapillary venule to venule to vein). Based on their ultrastructure, the tumor vessels can be classified into nine categories: a) arteries and arterioles; b) nonfenestrated capillaries; c) fenestrated capillaries; d) discontinuous capillaries (sinusoids); e) blood channels without endothelial lining; f) capillary sprouts; g) postcapillary venules (giant capillaries); h) venules and veins; and i) arteriovenous anastomoses (shunts) [7]. Note that except for vessels of classes (e) and (f), the remaining vessel types are structurally similar to those found in a normal tissue. The vessels of

classes (e) and (f) are found in healing (granulation) tissue. A key difference between normal and tumor vessels is that the latter are dilated, saccular and tortuous, and may contain tumor cells within the endothelial lining of the vessel wall [7]. In addition, unlike a normal tissue with a fixed route between arterial and venous sides, a tumor may have blood flowing from one venule to another via vessels of classes (b) through (g), or directly via an arteriovenous shunt. The branching patterns of blood vessels in a tumor are significantly different from those in a normal tissue, with many trifurcations, self-loops and spouts [8]. Furthermore, due to the peculiar nature of the vasculature, the organization of vessels may be different from one location to another and from one time to the next. As a result, one would expect different routes for blood flow in the well perfused advancing zone, semi-necrotic zone, and necrotic zone (Fig. 1).

Following the pioneering studies of Algire [9], several investigators have measured morphometric parameters of vessels in thin, two-dimensional tumors grown in transparent windows. The pioneering work of Gullino and Grantham [10] led to similar studies in three-dimensional experimental and human tumors [7]. The vascular space in tumors varies from 1% to 20% depending upon the tumor type, weight and method of measurement. Studies in two-dimensional tumors show that vascular volume, length and surface area increase during the early stages of growth, and then decrease; this behavior correlates with the onset of necrosis. The frequency of large diameter vessels increases in the later stages of growth. Most quantitative studies in three-dimensional tumors miss the early growth period of increase in vascular volume, length and surface area. While studies of later stages of growth show an increase in the intercapillary distance and a decrease in vessel length and surface area, the results on vascular volume are inconclusive. Some studies show that the fractional vascular volume of tumors remains fairly constant during growth (suggesting an increase in the number of blood vessels with sluggish flow), while others show that the fractional vascular volume decreases as a tumor grows (in agreement with the observation that tumor perfusion rate decreases as a tumor grows) [7]. Possible reasons for this discrepancy include errors associated with different measurement techniques as well as presence of arteriovenous shunts and blood vessels with stagnant blood in them. Whether the vascular volume decreases or not, a reduction in vascular surface area would lead to a reduction in the transvascular exchange of molecules. In addition, an increase in the intercapillary distance would require the molecules to traverse longer distances in the interstitium to reach all regions of a tumor.

*Blood flow rate*

Most investigators have measured local blood flow rate of tumors based on uptake or clearance of a tracer from a single or a limited number of regions of the tumor. Due to noticeable spatial and temporal heterogeneity in tumor blood supply, these values may not be representative of the whole tumor. A limited number of studies in which the blood flow rate of the whole tumor has been measured, shows that the *average* perfusion rate of carcinomas is less than that of the host tissue of origin. Sarcomas and lymphomas have higher *average* perfusion rates than carcinomas [11]. The data on blood flow in human tumors is limited and inconclusive due to methodological problems [12]. In general, as tumors grow larger, they may develop necrotic foci, and as a result, the average perfusion rate decreases with tumor size [11]. Note that even in these large necrotic tumors, therapeutic agents would be delivered in the well perfused regions.

Since the seminal work of Ide *et al.* [13], several investigators have examined the microscopic flow heterogeneities of tumors grown in transparent windows. Blood flow in tumor vessels has been found to be intermittent. There are random periods of flow reduction and stasis followed by resumption of flow, sometimes in the opposite direction [14, 15]. These fluctuations may result from a) vasomotor activity of the host arterioles, b) respiratory or cardiac cycle, c) rheological factors such as passage of red blood cells, white blood cells or cancer cells in a vessel, d) low perfusion pressures

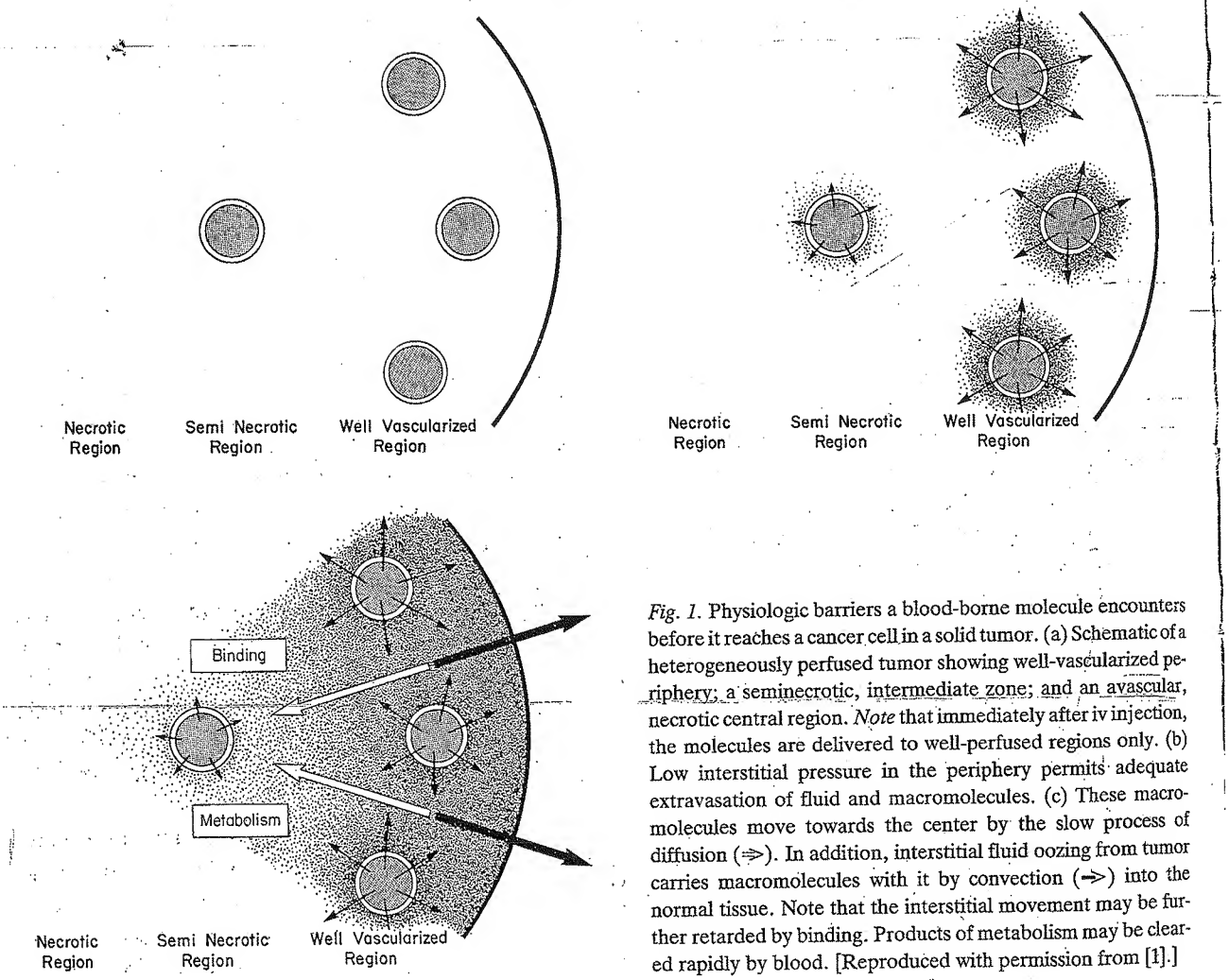


*Fig. 1.* Physiologic barriers a blood-borne molecule encounters before it reaches a cancer cell in a solid tumor. (a) Schematic of a heterogeneously perfused tumor showing well-vascularized periphery; a seminecrotic, intermediate zone; and an avascular, necrotic central region. *Note* that immediately after iv injection, the molecules are delivered to well-perfused regions only. (b) Low interstitial pressure in the periphery permits adequate extravasation of fluid and macromolecules. (c) These macromolecules move towards the center by the slow process of diffusion (⇒). In addition, interstitial fluid oozing from tumor carries macromolecules with it by convection (→) into the normal tissue. Note that the interstitial movement may be further retarded by binding. Products of metabolism may be cleared rapidly by blood. [Reproduced with permission from [1].]

in tumor vessels, and/or e) elevated interstitial pressure in tumors [7, 16, 17].

Quantitative studies on the macroscopic spatial heterogeneities in the tumor perfusion rate as a function of tumor growth (size) are limited. Based on perfusion rates four regions can be recognized in a tumor: a) an avascular, necrotic region, b) a seminecrotic region, c) a stabilized microcirculation region and d) an advancing front. In a rhabdomyosarcoma grown in the transparent chamber in a rat, the widths of the stabilized region and the advancing front were found to remain constant, while the widths of the necrotic and the seminecrotic zones increased with tumor growth. In addition, the perfusion rate in the tumor periphery (i.e., the stabilized and advancing zones) was found to be higher than that in the surrounding normal tissue [14]. Intratumor blood flow distributions in spontaneous animal and human tumors are now being investigated using nuclear magnetic resonance and positron emission tomography. While limited, these results are in concert with the transplanted tumor studies: blood flow rates in necrotic/seminecrotic regions of tumors are low, while those in non-necrotic regions are variable and substantially higher than in surrounding/contralateral host normal tissues [18, 19]. As a result of these spatial and temporal heterogeneities in blood supply coupled with variations in the vascular morphology at both macroscopic and microscopic levels, it is not sur-

prising that the spatial distribution of therapeutic agents in tumors is heterogeneous and the average uptake decreases with an increase in tumor weight.

## Transport across microvascular wall

Once a blood-borne molecule has reached an exchange vessel, its extravasation, $J_S$ (g/s), occurs by diffusion and convection (Fig. 2) and, to some extent, by transcytosis. Diffusion is proportional to the exchange vessel's surface area, S ($cm^2$), and the difference between the plasma and interstitial concentrations ($C_p$–$C_i$; g/ml). Convection is proportional to the rate of fluid leakage, $J_F$ (ml/s), from the vessel. $J_F$, in turn, is proportional to S and the difference between the vascular and interstitial hydrostatic pressures ($p_v$–$p_i$; mm Hg) minus the difference between the vascular and interstitial osmotic pressure ($\pi_v$–$\pi_i$; mm Hg). The proportionality constant which relates transluminal diffusive flux to concentration gradients ($C_p$–$C_i$) is referred to as the vascular permeability, P (cm/s), and the constant which relates fluid leakage to pressure gradients is referred to as the hydraulic conductivity, $L_p$ (cm/mm Hg-s). The effectiveness of the transluminal osmotic pressure difference in producing fluid movement across a vessel wall is characterized by the osmotic reflection coefficient, σ; σ is close to 1 for a macromolecule and close to zero for a small molecule [20]. Thus, transport of a molecule across normal or tumor vessels is governed by three transport parameters, P, $L_p$ and σ; the surface area for exchange, S; and the transvascular concentration and pressure gradients.

### *Transvascular transport parameters*

For a macromolecule of specified size, charge, configuration and binding constants, the transport parameters depend upon the physiological properties of the vessel wall (e.g., wall structure, charge). Ultrastructural studies of animal and human tumors have shown that tumor vessels have wide interendothelial junctions, large number of fenestrae and transendothelial channels formed by vesicles, discontinuous or absent basement membrane and a significant spatial heterogeneity [20, 21]. These characteristics of tumor vessels suggest that they should have relatively high P and $L_p$ [22, 23]. As a matter of fact, various tissue uptake studies have found vascular permeability of tumors to be significantly higher than that of skin or muscle (Fig. 3; [20]). If tumor vessels are indeed 'leakier' to fluid and macromolecules compared to several normal tissues, what leads to their poor extravasation? As discussed below, tumors contain regions of high interstitial pressure, which lowers the fluid extravasation. Since the transvascular transport of macromolecules under normal conditions occurs primarily by convection [20], a decrease in fluid extravasation would lead to a decrease in extravasation of macromolecules [25–27]. Furthermore, the average vascular surface area decreases with tumor growth, hence one would expect reduced transvascular exchange in large tumors compared to smaller tumors [27].

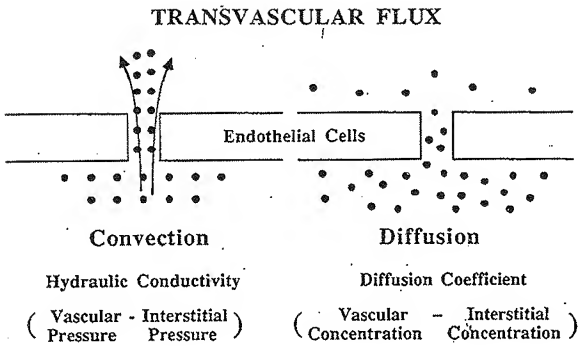


*Fig. 2.* Two primary modes of transvascular transport of molecules. Diffusion is proportional to concentration gradients and convection is proportional to pressure gradients. For more details on other modes of transport, see the text and [20].

### *Transvascular pressure gradients*

Decreased $p_v$ and/or increased $p_i$ in tumors has been indirectly demonstrated by several investigators working with tumors grown in transparent chambers. By raising venous pressure in the cham-

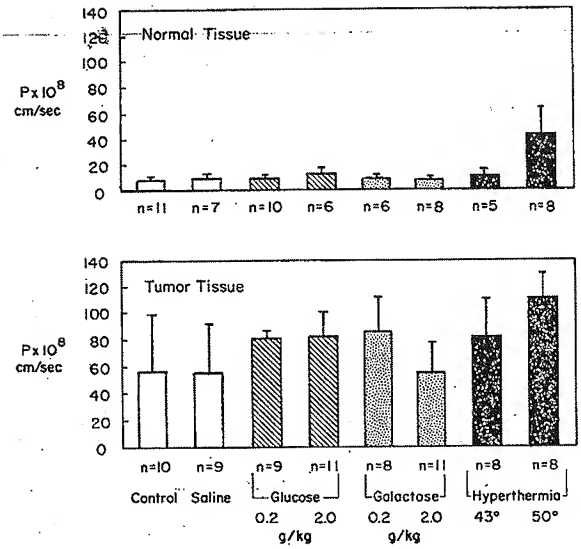


*Fig. 3.*Effective microvascular permeability coefficient of 150,000-molecular-weight dextran in normal (mature granulation) and neoplastic (VX2 carcinoma) tissues under various conditions: (a) control [37]; (b) after saline (1 ml/kg of body weight) injection; (c) after glucose injections; (d) after galactose injections; and (e) after hyperthermia for 1 hr [23] (mean ± SD; n = No. of measurements). *Note* that these measurements were made in *individual* blood vessels, and that tumor exchange vessels exhibit tremendous heterogeneity in vascular permeability. In addition, IgGs may extravasate at rates different from dextrans (Claus M, Jain RK, unpublished results). [Reproduced with permission from [24].]

ber or by loosening the chamber, blood flow can be restored in ischemic/necrotic tumor areas. Direct measurements in sandwich tumors or in the superficial layer of three-dimensional tumors have shown that on the arterial side vascular pressure does not differ significantly between non-tumor and tumor vessels; whereas, venous pressures may be lower in tumor vessels compared to those in normal vessels [7].

Since the initial work of Young *et al.* [28], several investigators have shown that $p_i$ in animal tumors is significantly higher than in normal tissues [29]. Recently, we have also quantified interstitial hypertension in human tumors. [For example, in superficial melanomas $p_i$ is as high as 45 mm Hg;[1] in cervical carcinomas as high as 30 mm Hg.[2]] Further as the tumor grows, $p_i$ rises in some tumors, presumably due to the proliferation of tumor cells in a confined space, high vascular permeability, and the absence of functioning lymphatic vessels [29–32]. This increase in $p_i$ also correlates with a reduction in tumor blood flow and the development of necrosis in a growing tumor [29]. Recent investigations of intratumor pressure gradients show that the interstitial pressure is elevated throughout the tumor and its drops precipitously to normal physiological values in the tumor periphery (Fig. 4; [30]). This pressure profile is in agreement with the predictions of our mathematical model [25–27].

In normal tissues $\pi_v$ and $\pi_i$ are approximately 20–25 and 5–15 mm Hg, respectively [25, 26]. Although there are no direct measurements of $\pi_i$ in tumors, based on high vascular permeability and high interstitial diffusion coefficient in tumors, one would expect higher concentration of endogenous plasma proteins in the tumor interstitium than in normal interstitium. This hypothesis is supported by the data in the literature [33]. As a result, $\pi_i$ in tumors may be higher than that in normal tissues, and may lead to reduced osmotic flow.

As shown in Fig. 4, $p_i$ in tumors is close to zero in the periphery, therefore the filtration of fluid from vessels, $J_F$, would be close to normal. However, as one moves towards the center of the tumor, the increase in $p_i$ would reduce the extravasation of fluid, $J_F$. As stated earlier, convective transport of a macromolecule is proportional to $J_F$, therefore, the rate of extravasation of a blood-borne macromolecule would be negligible in the center of a tumor [25, 26]. Since transvascular transport by diffusion is negligible for a macromolecule to begin with, macromolecular extravasation would be very small in the high interstitial pressure regions of a tumor. Since high pressure regions usually coincide with regions of poor perfusion rate and lower vessel surface area, leakage of blood-borne macromolecules from vessels would be further restricted [27].

## Transport through interstitial space

Once a macromolecule has extravasated, its movement occurs by diffusion and convection through the interstitial space [34]. Diffusion is proportional to the concentration gradient in the interstitium, and convection is proportional to the interstitial

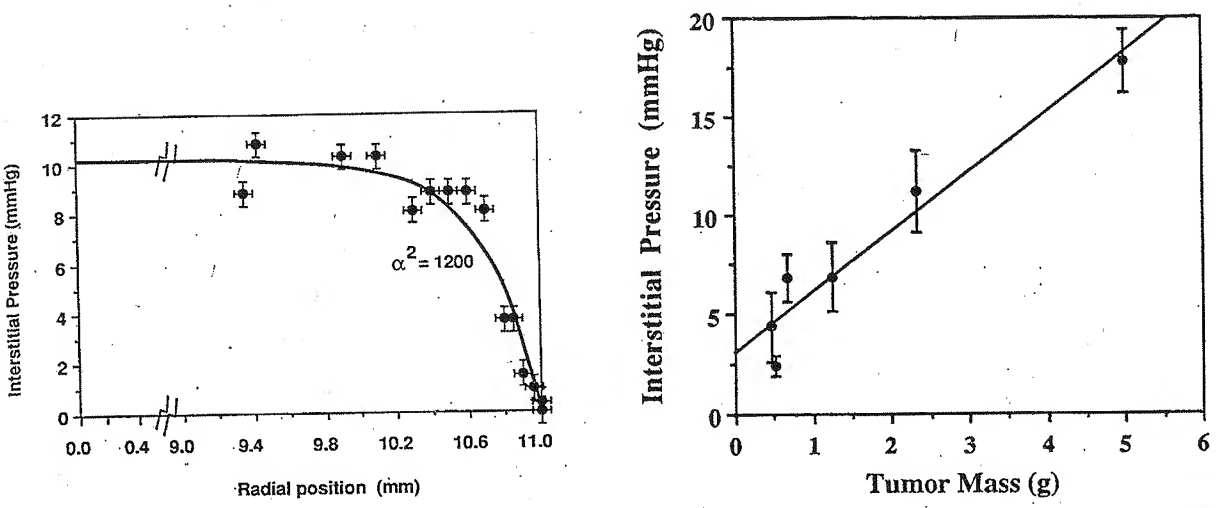


*Fig. 4.* (a) Interstitial pressure gradients in the mammary adenocarcinoma R3230AC as a function of radial position. The circles (●) represent data points [30] and the solid line represents the theoretical profile based on our previously developed mathematical model [25, 26]. *Note* that the pressure is nearly uniform in most of the tumor, but drops precipitously to normal tissue values in the periphery. (b) In Walker 256 carcinoma grown as tissue-isolated tumor, the mean central pressure (± SD) is linearly related to the tumor weight (pressure = 3.05 × weight (g) + 3.02 mm Hg). However, not all tumor types exhibited such correlation. In some tumors, the interstitial pressure increased initially with tumor growth and reached a maximum value (Belton A, Jain RK, unpublished results). We have also measured interstitial pressure as high at 45 mm Hg in human tumors (see text). *Note* that elevated pressure in the central region retards the extravasation of fluid and macromolecules. In addition the pressure drop from the center to the periphery leads to an experimentally verifiable, radially outward fluid flow. [Reproduced from [30], with permission.]

fluid velocity, $u_i$ (cm/s). The latter, in turn, is proportional to the pressure gradient in the interstitium. The proportionality constant which relates diffusive flux to the concentration gradient is referred to as the interstitial diffusion coefficient, D ($cm^2/s$), and the constant which relates $u_i$ to the pressure gradient is referred to as the interstitial hydraulic conductivity, K ($cm^2$/mm Hg-s) [29]. Values of transport coefficients D and K are determined by the structure and composition of the interstitial compartment as well as the physicochemical properties of the solute molecule. Larger values of these parameters lead to less hindered movement of fluid and macromolecules through the interstitium. Similarly, large values of interstitial pressure and concentration gradients lead to large convective and diffusive fluxes.

### *Interstitial transport coefficients*

The interstitial space in tumors, in general, is very large compared to that in host normal tissues [29]. Similar to normal tissues, the interstitial space of tumors is composed predominantly of a collagen and elastic fiber network. Interdispersed within this cross-linked structure are the interstitial fluid and macromolecular constituents (polysaccharides) which form a hydrophilic gel. While collagen and elastin impart structural integrity to a tissue, the polysaccharides (glycosaminoglycan and proteoglycans) are presumably responsible for the resistance to fluid and macromolecular motion in the interstitium. In several tumors studied to date, collagen content of tumors is higher than that of the host normal tissue. On the other hand, hyaluronate and proteoglycans are, in general, present in lower concentrations in tumors than in the host normal tissue [29]. The lower concentration of these polysaccharide molecules is presumably due to increased activity of lytic enzymes, e.g., hyaluronidase, in the tumor interstitial fluid [5].

The large interstitial space and low concentrations of polysaccharides suggest that values of K and D should be relatively high in tumors. As a matter of fact, the data on hydraulic conductivity of

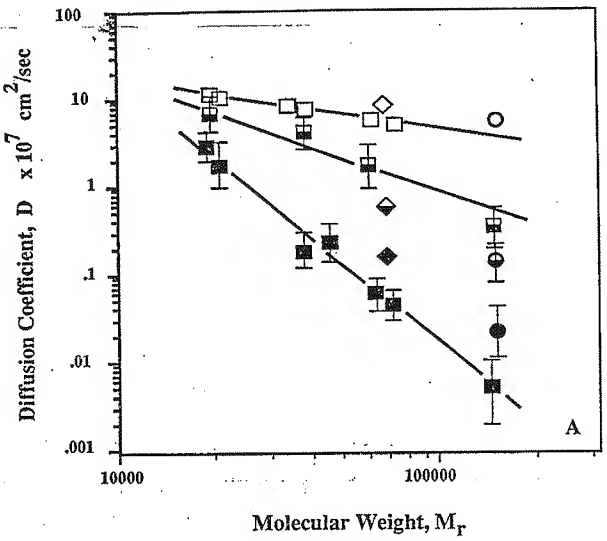


*Fig. 5.* Molecular weight dependence of effective diffusion coefficients, D, of dextrans [36, 37], bovine serum albumin [36] and rabbit IgG [38] in water, tumor, and normal (mature granulation) tissue. *Note* that transport is hindered in both tissues compared with water and that there is tremendous heterogeneity in both tissue types. Despite higher values of D in tumors compared with nontumor tissues, macromolecules do not reach uniform concentration in a large tumor for a long time because of large diffusion distances and large size of molecules. *Note* that the proteins show the same trend, i.e., the interstitial diffusion coefficient is higher in normal tissue and lower in tumor tissue than the corresponding molecular weight dextrans. The symbols represent:

□ Dextran-Water ◇ Albumin-Water ○ IgG-Water
◨ Dextran-Tumor ◈ Albumin-Tumor ◒ IgG-Tumor
■ Dextran-Normal ◆ Albumin-Normal ● IgG-Normal

[Reproduced from [36] with permission.]

hepatoma 5123 [35] and the data on effective diffusion coefficients of various macromolecules in VX2 carcinoma (Fig. 5) [36–38] support this hypothesis. An order of magnitude higher values of D and K in tumors compared to several normal tissues should favor movement of macromolecules in the tumor interstitium. Then, why do the exogenously injected macromolecules not distribute uniformly in tumors? As discussed below, there are two reasons for this apparent paradox:

*Large distances in the interstitium*

The time constant, $\tau_D$, for a molecule, with diffusion coefficient D, to diffuse across distance $l$ is approximately $l^2/4D$. For diffusion of IgG in tumors (using D from Fig. 5), $\tau_D$ is of the order of 1 hour for 100 $\mu$m distance, ~2 days for 1 mm distance and ~7–8 months for 1 cm distance [25, 38] (Fig. 6). These numbers are consistent with the data on the penetration of MAbs in spheroids [39]. Now consider a hypothetical tumor which is uniformly perfused, has nearly zero $p_i$, and has exchange vessels ~200 $\mu$m apart. In such a tumor, IgG would reach uniform concentration in ~1 hour post injection provided the plasma concentration remains constant. In a normal tissue with the value of D lower by an order of magnitude (Fig. 5), it will take ~10 hours to reach ~16% concentration.

Now consider a more realistic situation, where the tumor vessels are ~200 $\mu$m apart and uniformly perfused, but $p_i$ has increased in the center so that fluid extravasation, and, hence, convective transport of macromolecules across vessels has stopped. In such a case the only way macromolecules extravasate in the center is by the slow process of diffusion across vessel walls. Also they can reach the center from the periphery (where $p_i$ is near zero) by interstitial diffusion. As stated earlier, if the distance between the center and periphery is ~1 mm, it would take days for them to get there and if it is ~1 cm, it would take months [25, 38]. If due to elevated $p_i$ and cellular proliferation, the central vessels have collapsed completely, then there is no delivery of macromolecules by blood flow to the necrotic center [7]. In such a case, there are no molecules available for extravasation by diffusion across the vessel wall, and consequently the central concentration would be even lower [27].

So far the interstitial movement of molecules which do not bind to any extravascular sites or undergo metabolism has been discussed. It is well known that the binding reaction lowers the apparent diffusion rate of molecules [40]. Therefore, although higher affinity of antibody to antigen significantly increases the antibody's concentration proximal to the vessel, it retards their movement to

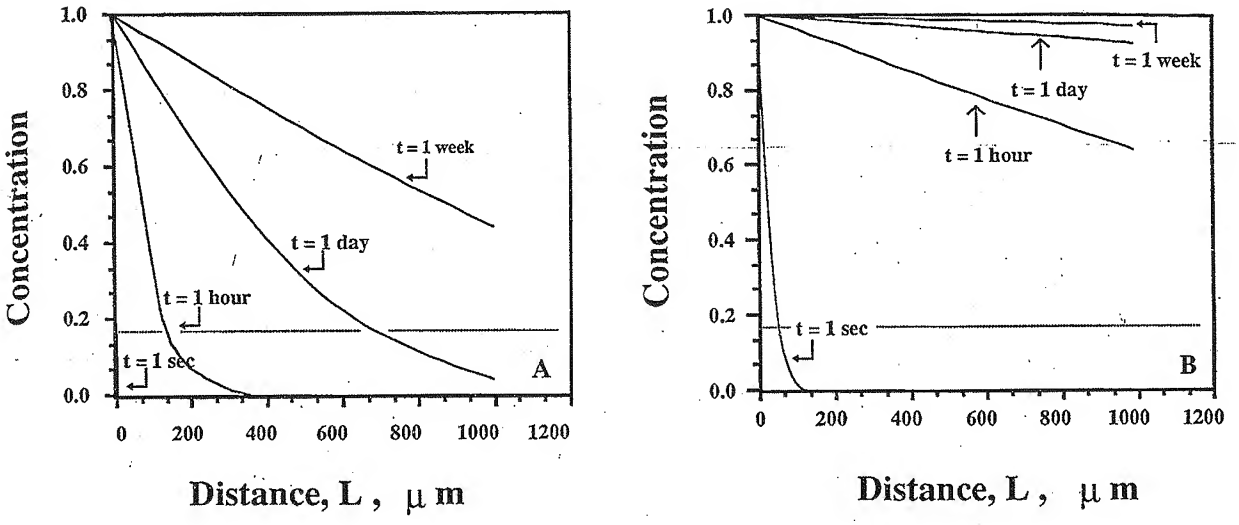


*Fig. 6.* The concentration profile of (a) IgG and (b) a low molecular weight agent ($M_r = 400$; $D_{tumor} = 6.4 \times 10^{-6}$ cm²/sec [36]) at various distances as a function of time into the tumor interstitium using a one-dimensional model. These profiles assume a constant source of the drug outside the blood vessel (L = 0). *Note* that in a short time (less than an hour) a large fraction of the low $M_r$ molecules has diffused to distances of 1 mm. On the other hand, for IgG, the fraction of antibody arriving at this point in 1 hour is nearly zero. The horizontal dotted lines show the time required to reach ~ 16% of the source concentration at distance L from the vessel wall. [Reproduced from [36] with permission.]

distal locations in the interstitium unless the antigens are saturated [41–44]. The metabolism of antibodies in normal and tumor tissues is poorly understood. However, the products of metabolism are usually smaller in molecular weight, and hence may be cleared relatively rapidly [43].

*Interstitial fluid loss from tumor's periphery*

It is a well known law of physics that fluid flows from a high to a low pressure region. As discussed earlier, $p_i$ is high in the center of tumors and low in the periphery. Therefore one would expect interstitial fluid motion from the center of a tumor towards its periphery from where it will ooze out into the surrounding normal tissue. Using a tissue isolated tumor preparation, Butler *et al.* [45] measured this fluid loss to be 0.14–0.22 ml/h/g-tissue in four different rat mammary carcinomas. In various animal and human (xenograft) tumors studied to date, 1–14% of plasma entering the tumor has been found to leave from the tumor's periphery [22, 45–47]. This fluid leakage leads to a radially outward interstitial fluid velocity of 0.1–0.2 μm/s at the periphery of a 1 cm 'tissue-isolated' tumor [20]. [The radially outward velocity is an order of magnitude lower in a tumor grown in the subcutaneous tissue or muscle [25–26].] A macromolecule at the tumor periphery has to overcome this outward convection to penetrate into the tumor by diffusion. The relative contribution of this mechanism of heterogeneous distribution of antibodies in tumors is, however, smaller than the contribution of heterogeneous extravasation due to elevated pressure and necrosis [25–27, 43].

**Conclusions and strategies for improved delivery**

Antibodies linked to radionuclides, drugs, toxins, enzymes, growth factors and effector cells offer a promising approach to the treatment of solid tumors. Their strenghts include their high degree of specificity for tumor-associated antigens and the fact that exchange vessels and interstitium of tumors are more 'leaky' to macromolecules than those of several normal tissues. Their clinical limitation, however, results from their inadequate uptake and non-optimal distribution in tumors. The

physiological factors which contribute to the poor delivery in tumors include – heterogeneous blood supply, interstitial hypertension, and relatively long transport distances in the interstitium (Table 1). How can these physiological barriers be overcome?

Several physical (e.g., radiation, heat) and chemical (e.g., vasoactive drugs) agents may lead to an increase in tumor blood flow or vascular permeability [7, 11, 48]. A key problem with this approach is that the increase in blood flow is short-lived and usually confined to well-vascularized regions. Increased delivery of macromolecules to well-perfused regions may not solve the maldistribution problem. However, an increase in the diffusive component of vascular permeability caused by these agents may increase the antibody uptake [49, 50].

The second approach may be based on lowering the tumor interstitial pressure. The interstitial hypertension results presumably from interstitial fluid accumulation which, in turn, results from the lack of functioning lymphatics in tumors [5, 25, 29, 30]. Since K is a key determinant of interstitial fluid motion, any method which increases K may lower pressure. Use of lytic enzymes (e.g., hyaluronidase) to increase K is one possibility [35]. An alternate strategy would be to lower the tumor cell density without destroying the vasculature. Whether fractionated radiation or other therapies (e.g. TNF) lowers $p_i$ in tumors via this mechanism remains a plausible hypothesis to be tested [20, 25]. [In support of this hypothesis, we have recently found that in human cervical carcinomas, the pressure decreased during fractionated radiation treatment in some patients.[2]] The use of an osmotic agent (e.g. mannitol) may increase $\pi_v$, and hence increase antibody penetration [51]. However, this increase must have a long duration to yield practical results.

The third approach may be based on increasing the interstitial transport rate of molecules. Use of cocktails of antibodies may not overcome this problem because each antibody has to cross the same physiological barriers. One method of accomplishing this goal would be to use lower molecular weight agents, e.g., antibody fragments $F_{(ab')_2}$ and $F_{ab}$. While the fragments have higher values of P and D compared to the intact antibody and hence, penetrate deeper into tumors, there are two physiological problems associated with their use – they are eliminated more rapidly from blood, and their uptake into normal tissues is also increased. The elimination problem can be overcome by repeated or continuous injections of non-immunogenic fragments of chimeric or human antibodies. However, as the molecular weight is lowered further, the normal tissue toxicity problem may become more pronounced similar to that encountered with conventional anticancer agents (molecular weight $< 2{,}000$) [52, 53]. Some of the problems with the systemic toxicity may be overcome by local injection (e.g., intra-arterial, interstitial, intraperitoneal) at the cost of not being able to reach the distant metastases. If the toxicity to normal tissue could be overcome, combination of local and systemic injections would be more effective. Similarly, delivering low molecular weight agents (e.g.,

*Table 1.* Physiological promises and problems in the delivery of macromolecules to tumors

| |
|---|
| Promises |
| • Relatively high degree of specificity of antibodies for tumor-associated antigens |
| • Relatively large vascular permeability, interstitial diffusion coefficient, and hydraulic conductivity |
| Problems |
| • Heterogeneous blood supply |
| • Elevated interstitial pressure |
| • Fluid loss from periphery |
| • Large distances in the interstitium |
| • Large affinity and heterogeneous binding |
| • Metabolism |

drug, toxin, enzyme, hormone) linked to MAbs and releasing them once they have extravasated or entered cells seems reasonable. However, once a small molecule is uncoupled from the antibody it may diffuse back into a nearby blood vessel, and may be rapidly eliminated.

Ideally, an antibody should have a high specificity and low molecular weight. To this end, recent developments in producing recombinant DNA monoclonal antibodies have already yielded smaller antibody fragments (e.g., antibody binding site, molecular recognition unit). In addition, two other approaches seem to satisfy the requirement of low molecular weight with increased specificity: the use of low molecular weight chelates with bifunctional antibodies (BFA) [54] and the use of low molecular weight prodings with enzyme-conjugated antibodies (ECA) [55]. In these two-step approaches, BFA (or ECA) is injected into a patient, permitted to bind to antigenic sites in the tumor, and then cleared from the normal tissues. At an appropriate time later, a radionuclide attached to a low molecular weight chelate (or a prodrug) is injected into the patient with the advantage of rapid delivery to the tumor and clearance from the body. An increase in antigen expression and/or affinity may help this approach. The number of antigenic sites may be increased using biological response modifiers such as interferon [56]. This would increase the concentration of antibody near the blood vessels, but would not increase the depth of penetration until the antigens are saturated [43, 44]. One way of overcoming some of the problems of poor antibody localization is to use radioisotopes with large tumor dose deposition and large depths of penetration; however toxicity to normal tissues may become a limiting factor. Protecting bone marrow using growth factors (e.g., interleukin-1, colony-stimulating factors) or with bone marrow transplant may alleviate the normal tissue toxicity problem. Another method is to combine antibody treatment with other modalities (e.g., radiation sensitizers, low molecular weight cytotoxic drugs to synchronize cell cycle, hyperglycemia to lower pH [57]) depending upon the tumor type. Finally, antibodies directed against the necrotic tissue may exploit the 'reservoir' phenomenon seen in tumors [25–27, 43, 52].

In contrast, the physiological barriers discussed in this article may not be a problem for: (i) radioimmunodetection, (ii) treating leukemias, lymphomas and small tumors (e.g., micrometastases) in which the interstitial pressure is low and diffusion distances are small, (iii) treatment of adequately perfused, low pressure regions of large tumors [58], and (iv) treatment with antibodies directed against the tumor endothelial cells or microenvironment of the subendothelial matrix. We have recently shown that activation with lymphokines increases the rigidity of LAK cells [59]. These results suggested that the uptake of LAK cells might be increased by intra-arterial injection of these cells into an organ infiltrated by tumor metastases [59]. We have indeed found this to be true in VX2 carcinoma grown in rabbits [60]. These results suggest that in addition to the direct cytotoxicity against cancer cells, the LAK cell therapy may be vasculature mediated [60]. Thus LAK cells may be useful as drug carriers to the solid tumors [60, 61], and may complement anti-angiogenic therapeutic approaches [6]. In fact, the tumor vasculature and the subendothelial matrix may be a target for several conventional and novel therapies (e.g., TNF, hyperthermia, anti-angiogenic factors), and hence must be exploited in cancer treatment.

These physiological barriers may also not pose any problems for treatment with a molecule or cell which has nearly 100% specificity for cancer cells. Until such molecules or cells are developed, methods are urgently needed to overcome or exploit these physiological barriers in tumors. It is hoped that an improved understanding of tumor physiology will help in developing these strategies. In the meantime, the search for highly selective, tumor-specific agents must go on using *in vitro* methodologies. Once a novel therapeutic agent has been identified based on extensive *in vitro* screening, we need to ask whether this agent will arrive in all regions of a tumor, and more importantly, whether it will arrive there in therapeutically adequate quantities with minimal toxicity to normal tissues. Only after the answers to these questions is positive may the agent be considered as a breakthrough. It

is also hoped that the physiological insight presented in this article helps in answering these questions and subsequently becomes a prerequisite to the optimal development of novel therapeutic strategies for treatment of solid tumors including the development of anti-angiogenic modalities as well as the delivery of effector cells (e.g., lymphokine activated killer cells, tumor infiltrating lymphocytes) to the tumor microenvironment.

## Key unanswered questions

- How different are the transport parameters of human tumors from those of animal tumors?
- How can the physiological barriers in tumors be overcome predictably and reproducibly using chemical or physical agents?
- What are the optimal size, charge, configuration and affinity of a molecule for cancer detection and treatment?
- How can the rigidity, adhesiveness and motility of activated lymphocytes be modulated to increase their localization in solid tumors?
- How can the physical and biological characteristics of the tumor vasculature and interstitium be exploited for cancer treatment?

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# TRANSPORT OF MOLECULES, PARTICLES, AND CELLS IN SOLID TUMORS

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■ **Abstract** Extraordinary advances in molecular biology and biotechnology have led to the development of a vast number of therapeutic anti-cancer agents. To reach cancer cells in a tumor, a blood-borne therapeutic molecule, particle, or cell must make its way into the blood vessels of the tumor and across the vessel wall into the interstitium, which it then must migrate through. Unfortunately, tumors often develop in ways that hinder these steps. The goal of research in this area is to analyze each of these steps experimentally and theoretically and integrate the resulting information into a unified theoretical framework. This paradigm of analysis and synthesis has fostered a better understanding of physiological barriers in solid tumors and aided in the development of novel strategies to exploit and/or overcome these barriers for improved cancer detection and treatment.

CONTENTS



## INTRODUCTION

Within 5 years, cancer may surpass cardiovascular diseases as the number one cause of death in the United States (96). Our nation's investment in cancer research has led to unprecedented insight into the molecular origins of cancer.

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These advances have helped to identify novel targets and develop a vast array of therapeutic agents. For these agents to be successful, they must satisfy two requirements: (*a*) the relevant agent must be effective in the in vivo microenvironment of tumors, and (*b*) this agent must reach the target cells in vivo in optimal quantities. The goal of research in this area is to examine the latter issue—the delivery of diagnostic and therapeutic agents to solid tumors and normal host tissues.

All conventional and novel therapeutic agents can be divided into three categories—molecules, particles, and cells. For example, in chemotherapy, the agent can be injected as a molecule or incorporated in a nano-particle or liposome. In gene therapy, it can be a molecule, a viral or nonviral particle, or a genetically engineered cell. In immunotherapy, it can be a molecule, such as an antibody, or a cell, such as activated lymphocytes.

A blood-borne molecule or particle that enters the tumor vasculature reaches cancer cells via distribution through the vascular compartment, transport across the microvascular wall, and transport through the interstitial compartment. For a molecule of given size, charge, and configuration, each transport process may involve diffusion and convection. In addition, during the journey the molecule may bind nonspecifically to proteins or other tissue components, bind specifically to the target(s), or be metabolized (69). Although lymphokine-activated killer cells (lymphocytes activated by the lymphokine interleukin-2) or tumor-infiltrating lymphocytes are capable of deformation, adhesion, and migration, they encounter the same barriers that restrict their movement in tumors. Some of these physiological parameters are also important for heat transfer in normal and tumor tissues during hyperthermic treatment of cancer (70).

The overall aim of research in this area is to develop a quantitative understanding of each of the above-mentioned steps involved in the delivery of various agents. More specifically, the goal is to understand (*a*) how angiogenesis takes place and what determines blood flow heterogeneities in tumors, (*b*) how blood flow influences the metabolic microenvironment in tumors and how microenvironment affects the biological properties of tumors (e.g. vascular permeability, cell adhesion), (*c*) how material moves across the microvascular wall, and (*d*) how material moves through the interstitial compartment and the lymphatics. In addition, (*e*) the role of cell deformation and adhesion in the delivery of cells has been examined. Finally, (*f*) knowledge of these processes for molecules, particles, and cells has been integrated into a unified framework for scale-up from mice to men (Figure 1; see color figure). In this article, I describe various experimental and theoretical approaches, recent findings in these six areas, and how some of these concepts have been taken from bench to bedside for potential improvement in cancer detection and treatment.

## EXPERIMENTAL AND THEORETICAL APPROACHES

The following five approaches have been used to gain insight into transport phenomena in solid tumors.

1. A tissue-isolated tumor connected by a single artery and a single vein to the circulation of the host (148, 149). This technique, originally developed in 1961 for rats (54), has recently been adapted to mice (101, 102) and humans (107).
2. A modified Sandison rabbit ear chamber (31, 169), a modified Algire mouse dorsal chamber (113, 114), and a cranial window in mice and rats (168). Use of the ear chamber offers the advantage of superior optical quality and using the mice offers the advantage of working with immunodeficient and genetically engineered cells and animals (23, 30, 42, 127, 161). A quantitative angiogenesis assay was recently developed using these transparent windows to study the physiology of vessels induced by individual growth factors (28, 79, 147) (Figure 2; see color figure). In addition, single vessels of tumors have been perfused in these windows (115, 116), several acute preparations, e.g. liver and mesentery, have been utilized (44), and a new model to visualize lymphatic and lymphangiogenesis in the mouse tail has been developed (86, 110).
3. In vitro methods to assess the deformability, adhesion, permeability, and growth stress of normal and neoplastic cells (58, 119, 129, 145, 158), as well as measurements of the expression of adhesion molecules in intact monolayers (77, 128) (Figure 3; see color figure).
4. Various molecular biology techniques (e.g. in situ hybridization and Southern, Northern, and Western blotting), including development of genetically engineered cells and mice (23, 30, 42, 86, 127, 161). Also, green fluorescence protein has been used as an in vivo reporter to monitor promoter activity noninvasively (42).
5. Mathematical models to describe and integrate the data obtained from the above four approaches, to scale up biodistribution data from mice to men, and to design future experiments (6–9, 11, 12, 37, 74, 82, 83, 132, 133, 135, 140, 163).

Each of these approaches has its limitations. In combination, however, they have permitted development of the framework for tumor microcirculation and drug delivery described in this article.

## DISTRIBUTION THROUGH VASCULAR SPACE

The chaotic blood supply of tumors is the first barrier encountered by a blood-borne agent. The tumor vasculature consists both of vessels recruited from the preexisting network of the host vasculature and of vessels resulting from the angiogenic response of host vessels to cancer cells (39, 67). Movement of mol-

ecules through the vasculature is governed by the vascular morphology (i.e. the number, length, diameter, and geometric arrangement of various blood vessels) and the blood flow rate (2, 4, 6, 49, 108).

Although the tumor vasculature originates from the host vasculature and the mechanisms of angiogenesis are similar (39, 108, 139), its organization may be completely different, depending on the tumor type, its growth rate, and its location. The fractal dimensions and minimum path lengths of tumor vasculature are different from those of the normal host vessels (2, 3, 48, 49). The architecture and blood flow are different not only among various tumor types but also between a tumor and its metastases (67, 81). For example, unlike in normal tissue, where the velocity of red blood cells is dependent on vessel diameter, there is no such dependence in tumors (44, 114, 168). Furthermore, the velocity of red blood cells may be an order of magnitude lower in some tumors compared with the host vessels (Figure 4). The temporal and spatial heterogeneity in tumor blood flow may, in part, be a result of elevated geometric and viscous resistance in tumor vessels (107, 149, 150, 151) coupling between high vascular permeability and elevated interstitial fluid pressure (4, 135), vascular remodeling by intussusception (139), and solid stress generated by proliferating cancer cells (53, 58).

Based on perfusion rates, four regions can be recognized in a tumor: an avascular, necrotic region; a seminecrotic region; a stabilized microcirculation region; and an advancing front (34) (Figure 5). Intratumor blood flow distributions in spontaneous animal and human tumors are now being investigated with nuclear magnetic resonance, positron emission tomography, and functional computed tomography (35, 37, 56, 153). Although limited, these results are in concert with the transplanted tumor studies: Blood flow rates in necrotic and seminecrotic regions of tumors are low, whereas those in nonnecrotic regions are variable and can be substantially higher than in surrounding (contralateral) host normal tissues (159). Considering these spatial and temporal heterogeneities in blood supply coupled with variations in the vascular morphology at both microscopic and macroscopic levels, it is not surprising that the spatial distribution of therapeutic agents in tumors is heterogeneous and that the average uptake decreases, in general, with an increase in tumor weight. This perfusion heterogeneity also makes it difficult to heat the tumor periphery during hyperthermia (70).

## METABOLIC MICROENVIRONMENT

The temporal and spatial heterogeneities in blood flow lead to a compromised metabolic microenvironment in tumors. To quantify the spatial gradients of key metabolites, two optical techniques were recently adapted: fluorescence ratio-imaging microscopy and phosphorescence quenching microscopy (27, 60, 117, 118, 157). Both pH and $pO_2$ decrease with distance from tumor vessels, leading to acidic and hypoxic regions in tumors (Figure 6). Coupled with the use of cells selected for impaired glycolytic and oxidative pathways, these methods have pro-

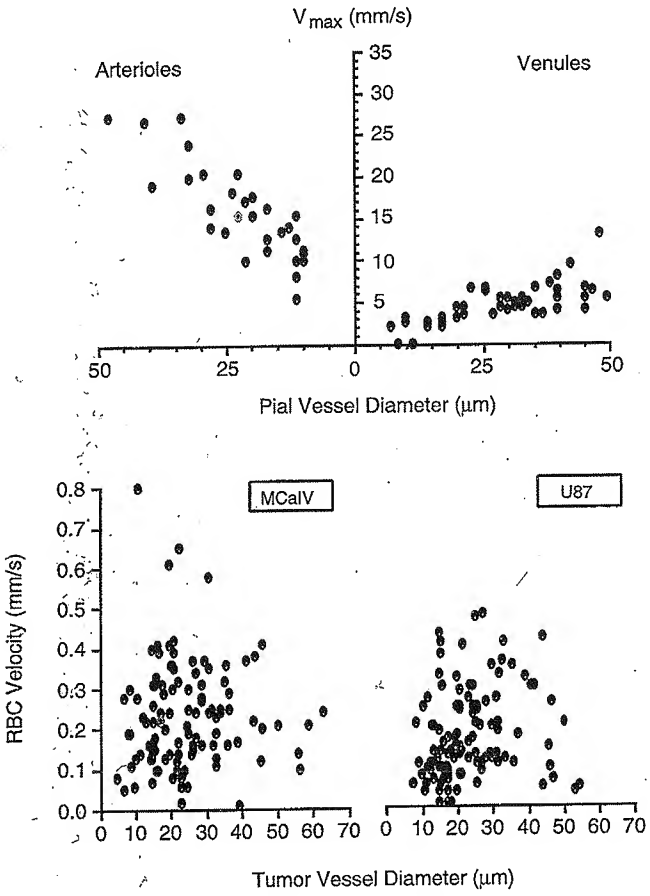


**FIGURE 4** Blood velocity as a function of vessel diameter in (*top*) normal pial vessels and (*bottom*) a murine mammary carcinoma (MCaIV) and a human glioma (U87) xenograft on the pial surface. Note that in normal microcirculation, blood velocity is dependent on vessel diameter, whereas in tumors there is no such dependence. Furthermore, the blood velocity in tumor vessels is about an order of magnitude lower than in host vessels. RBC, red blood cells. (Adapted from Reference 168.)

vided novel insight into pH regulation in tumors (59). Although low $pO_2$ and pH are detrimental to some therapies (e.g. radiation), they might enhance the effect of certain drugs, if the drug could be delivered in adequate quantities to those regions (80, 136, 160).

To gain further insight into tumor metabolism, two powerful approaches have been combined: magnetic resonance spectroscopy and tissue-isolated tumors. The former allows measurement of the energy level in tumors whereas the latter allows control of the supply of individual substrates (e.g. glucose, oxygen) to the tumor.

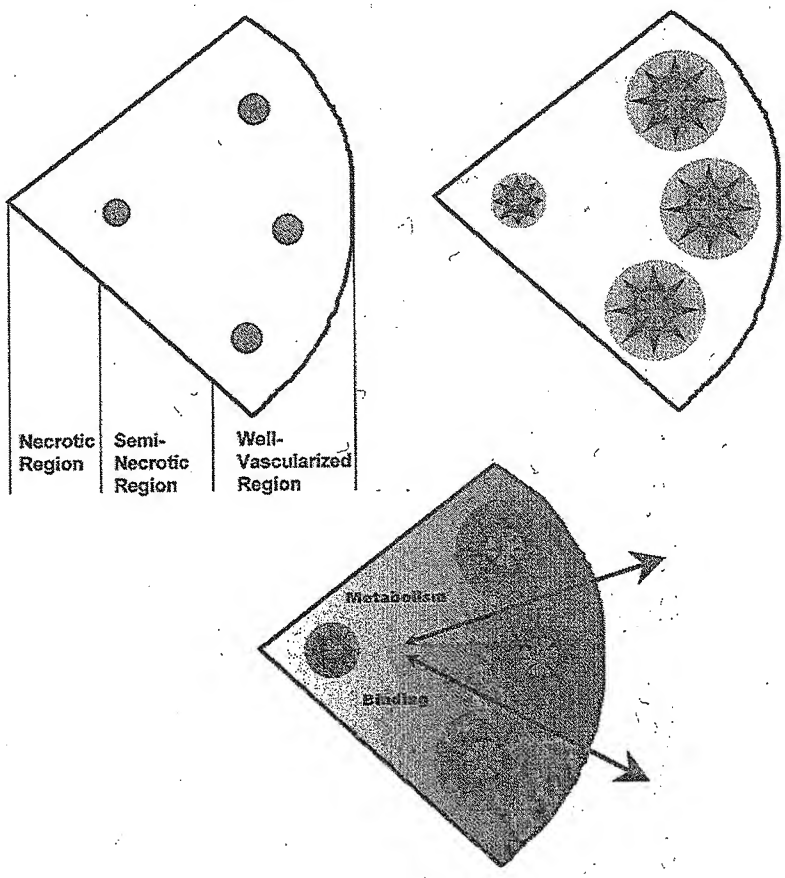


**FIGURE 5** Physiological barriers that a blood-borne molecule encounters before it reaches a cancer cell in a solid tumor. (*Top left*) Schematic of a heterogeneously perfused tumor showing well-vascularized periphery; a seminecrotic, intermediate zone; and an avascular, necrotic central region. Note that immediately after intravenous injection, the molecules are delivered to perfused regions only. (*Top right*) Low interstitial pressure in the periphery permits adequate extravasation of fluid and macromolecules. (*Bottom*) These macromolecules move toward the center by the slow process of diffusion. In addition, interstitial fluid oozing from tumor carries macromolecules with it by convection into the normal tissue. Note that the interstitial movement may be further retarded by binding. Products of metabolism may be cleared rapidly by blood. (Adapted from Reference 68.)

Using this approach, Eskey et al (36) recently showed that solid tumors depend more on glucose than oxygen to maintain their ATP level. Using a sandwich culture system, Helmlinger et al (57) are currently examining the relationship between the gradients of metabolites and gene expression. Two novel findings have resulted from this work on hypoxia. The relationship between hypoxia and

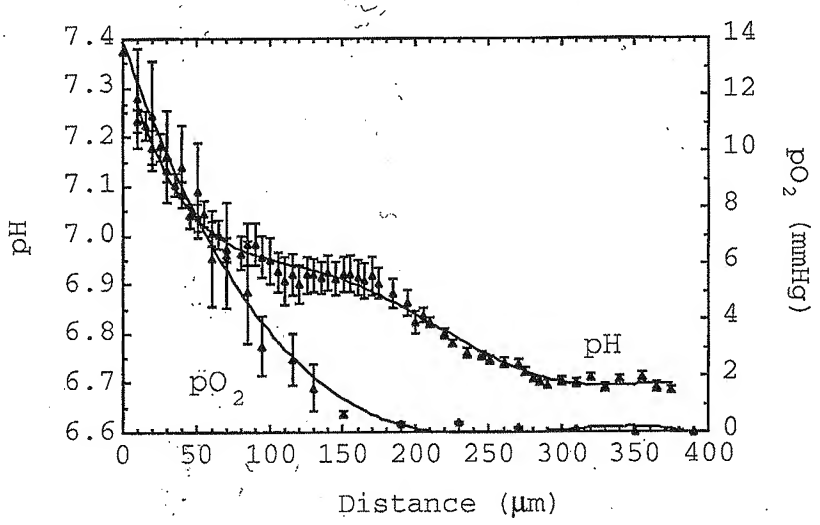


**FIGURE 6** Spatial gradients of metabolites in tumors. pH gradients measured using fluorescence ratio imaging microscopy. $pO_2$ gradients measured using phosphorescence quenching. Distance from the vessel wall, in microns, is shown on the x-axis, with zero being the vessel wall. (Adapted from Reference 60.)

vascular endothelial growth factor (VEGF) promoter activity in vivo is not as expected from in vitro studies. In addition, deletion of hypoxia-inducible factor 1-α lowers angiogenesis and oxygenation in tumors. Surprisingly, instead of growing slowly, these tumors grew faster (23).

## TRANSPORT ACROSS THE MICROVASCULAR WALL

Once a blood-borne molecule has reached an exchange vessel, its extravasation, $J_s$ (g/s), occurs by diffusion, convection and, to some extent, presumably transcytosis (65). Diffusive flux is proportional to the exchange vessel's surface area, $S$ ($cm^2$), and the difference between the plasma and interstitial concentrations, $C_p$–$C_i$ (g/ml). Convection is proportional to the rate of fluid leakage, $J_f$ (ml/s), from the vessel. $J_f$, in turn, is proportional to $S$ and the difference between the vascular and interstitial hydrostatic pressures, $p_v$–$p_i$ (mm Hg), minus the osmotic reflection coefficient ($\sigma$) times the difference between the vascular and interstitial osmotic pressures $\pi_v$–$\pi_i$ (mm Hg). The proportionality constant that relates transluminal diffusion flux to concentration gradients, ($C_p$–$C_i$), is referred to as the vascular permeability coefficient, $P$ (cm/s), and the constant that relates fluid leakage to pressure gradients is referred to as the hydraulic conductivity, $L_p$ (cm/mm Hg · s). The effectiveness of the transluminal osmotic pressure difference in producing fluid movement across a vessel wall is characterized by $\sigma$, which is

close to one for a macromolecule and close to zero for a small molecule. Thus, the transport of a molecule across normal or tumor vessels is governed by three transport parameters ($P$, $L_p$, and $\sigma$), the surface area for exchange, and the transvascular concentration and pressure gradients.

Vascular permeability and hydraulic conductivity of tumors in general are significantly higher than that for various normal tissues (33, 52, 65, 116, 152, 166–168), and hence, these vessels may lack permselectivity (165). Positively charged molecules have a higher permeability (29). Despite increased overall permeability, not all blood vessels of a tumor are leaky (Figure 7; see color figure). Even the leaky vessels have a finite pore size, which has been measured in a variety of human and rodent tumors (61). The hypothesis is that the large pore size in tumors represents wide interendothelial junctions (61, 143). Not only does the vascular permeability vary from one tumor to the next, but within the same tumor it varies both spatially and temporally, and during tumor growth, regression, and relapse (65, 78). The local microenvironment plays an important role in controlling vascular permeability. For example, a human glioma (HGL21) is fairly leaky when grown subcutaneously in immunodeficient mice, but it exhibits blood-brain barrier properties in the cranial window (Figure 7). Such site-dependent differences have been found with other tumors in other orthotopic sites (44). The working hypothesis is that the host–tumor interactions control the production and secretion of cytokines associated with permeability changes [e.g. vascular permeability factor (VPF)/VEGF and its inhibitors] (42, 84). A better understanding of the molecular mechanisms of permeability regulation in tumors is likely to yield strategies for improved drug delivery (164).

If tumor vessels indeed leak fluid and macromolecules, then what leads to the poor extravasation of these agents in various regions of tumors? Experimental and human tumors exhibit high interstitial fluid pressure (1, 16, 18–20, 22, 25, 55, 66, 106, 131, 144, 175, 176) (Table 1). Furthermore, the uniformly high pressure drops precipitously to normal values in the periphery of the tumor or in the peritumor region (5, 16, 74). This may lower fluid extravasation in the high-pressure regions, especially because oncotic and hydrostatic pressures are also equal between the intravascular and extravascular space (18, 21, 154). Because the transvascular transport of macromolecules in normal tissues occurs primarily by convection (65, 142), convective transport of macromolecules in the center of tumors may be less than in the tumor periphery (5, 74, 116). Additionally, the average vascular surface area per unit of tissue weight decreases with tumor growth; hence, reduced transvascular exchange would be expected in large tumors compared with small tumors (5, 6).

## TRANSPORT THROUGH INTERSTITIAL SPACE AND LYMPHATICS

Once a molecule has extravasated, its movement through the interstitial space occurs by diffusion and convection (66). Diffusion is proportional to the concentration gradient in the interstitium, and convection is proportional to the interstitial

**TABLE 1** Interstitial fluid pressure (mm Hg) in normal and neoplastic tissues in patients

| Tissue type | N[a] | Mean | Range |
|---|---|---|---|
| Normal skin | 5 | 0.4 | −1.0–3.0 |
| Normal breast | 8 | 0.0 | −0.5–3.0 |
| Head and neck carcinomas | 27 | 19.0 | 1.5–79.0 |
| Cervical carcinomas | 26 | 23.0 | 6.0–94.0 |
| Lung carcinomas | 26 | 10.0 | 1.0–27.0 |
| Metastatic melanomas | 14 | 21.0 | 0.0–60.0 |
| Metastatic melanomas | 12 | 14.5 | 2.0–41.0 |
| Breast carcinomas | 13 | 29.0 | 5.0–53.0 |
| Breast carcinomas | 8 | 15.0 | 4.0–33.0 |
| Brain tumors[b] | 17 | 7.0 | 2.0–15.0 |
| Brain tumors[b] | 11 | 1.0 | −0.5–8.0 |
| Colorectal liver metastasis | 8 | 21.0 | 6.0–45.0 |
| Lymphomas | 7 | 4.5 | 1.0–12.5 |
| Renal cell carcinoma | 1 | 38.0 | — |

[a]N, Number.
[b]Patients were treated with anti-edema therapy.

fluid velocity, $u_i$ (cm/s). The latter, in turn, is proportional to the pressure gradient in the interstitium. Just as the interstitial diffusion coefficient, $D$ (cm$^2$/s), relates the diffusive flux to the concentration gradient, the interstitial hydraulic conductivity, $K$ (cm$^2$/mm Hg · s), relates the interstitial velocity to the pressure gradient (66). Values of these transport coefficients are determined by the structure and composition of the interstitial compartment as well as by the physicochemical properties of the solute molecule (14, 24, 87–89, 137, 141, 155).

Using fluorescence recovery after photobleaching, Berk et al found $D$ of various molecules in neoplastic tissue to be about one-third that in water (15) and to be similar to that in the host tissue (24). Similarly, the value of $K$ for a human colon carcinoma xenograft (LS174T) measured using two different methods (17, 175) was found to be higher than that of a hepatoma (155), which in turn was higher than that of the liver. Given these relatively high values of $D$ and $K$, why are exogenously injected macromolecules not distributed uniformly in tumors? As discussed next, there are two reasons for this apparent paradox.

The time constant for a molecule with diffusion coefficient $D$ to diffuse across distance $L$ is approximately $L^2/4D$. For diffusion of immunoglobulin G in tumors, this time constant is 1 h for a 100-μm distance, days for a 1-mm distance, and months for a 1-cm distance. Thus, for a 1-mm tumor, diffusional transport would take days, and for a 1-cm tumor, it would take months. If because of cellular proliferation (58) and interstitial matrix rearrangement the central vessels have collapsed completely, there would be no delivery of macromolecules by blood

flow to this necrotic center (53). Binding may further retard the transport in tumors (7, 8, 15, 90–94). The role of binding is clearly illustrated in Figure 8 (see color figure), which compares the rate of fluorescence recovery of a photobleached spot in tumor tissue injected with both nonspecific and specific immunoglobulin G. In addition to the heterogeneity in $D$ in tumors, the most unexpected result of these photobleaching studies was the large extent (30%–40%) of nonspecific binding (15).

As mentioned earlier, interstitial fluid pressure is high in the center of tumors and low in the periphery and surrounding tissue (5, 16, 74). Therefore, one would expect interstitial fluid motion from the periphery of the tumor into the surrounding normal tissue (Figure 5). In various animal and human (xenograft) tumors studied to date, 6%–14% of plasma entering the tumor has been found to leave from the periphery of the tumor (65, 68). This fluid leakage leads to a radially outward interstitial fluid velocity of 0.1–0.2 μm/s at the periphery of a 1-cm tissue-isolated tumor (65). [The radially outward velocity is likely to be an order of magnitude lower in a tumor grown in the subcutaneous tissue or muscle (5).] A macromolecule at the tumor periphery has to overcome this outward convection to diffuse into the tumor. The relative contribution of this mechanism of heterogeneous distribution of antibodies in tumors may be smaller than the contribution of heterogeneous extravasation because of elevated pressure and necrosis (5).

In most normal tissues, extravasated macromolecules are taken up by the lymphatics and brought back to the central circulation. Because of the lack of functional lymphatics within the tumor, the fluid and macromolecules oozing from the tumor surface must be picked by the peritumor host lymphatics (7). To characterize the transport into and within the lymphatic capillaries, Leu et al (110) recently developed a mouse tail model. Uptake and transport in this model have been measured using a macroscopic approach (routine test dilution analysis) and a microscopic approach (fluorescence recovery after photobleaching) (13, 156). Current efforts are directed toward uncovering mechanisms of lymphangiogenesis (86) and understanding changes in lymphatic transport in the presence of a tumor (109), the working hypothesis being that proliferating tumor cells generate enough stress so that even if lymphatics form in tumors, they collapse.

## TRANSPORT OF CELLS

Thus far, discussion has been limited to the parameters that govern the transport of molecules and particles (e.g. liposomes) in tumors. When a leukocyte enters a blood vessel, it may continue to move with flowing blood, collide with the vessel wall, adhere transiently or stably, and finally extravasate. These interactions are governed by both local hydrodynamic forces and adhesive forces. The former are determined by the vessel diameter and fluid velocity, and the latter by the expression, strength, and kinetics of bond formation between adhesion molecules and by surface area of contact (125, 130). Deformability of cells affects both types

of forces. Despite their importance in immunotherapy and gene therapy, the determinants of cell transport in tumors have not been examined.

Using intravital microscopy, Fukumura et al (41) recently showed that rolling of endogenous leukocytes is generally low in tumor vessels, whereas stable adhesion (≥30 s) is comparable between normal and tumor vessels. On the other hand, both rolling and stable adhesion are nearly zero in angiogenic vessels induced in collagen gels by basic fibroblast growth factor (bFGF) or VEGF/VPF, two of the most potent angiogenic factors (28). Whether the latter is due to a low flux of leukocytes into angiogenic vessels and/or down-regulation of adhesion molecules in these immature vessels is currently under investigation. The age of the animal also plays an important role in leukocyte-endothelial interactions (162).

To gain further insight into the types of cells that adhere to tumor vessels, the localization of interleukin-2–activated natural killer (A-NK) cells in normal and tumor tissues in mice was examined using positron emission tomography (119, 120). Immediately after systemic injection, these cells were localized primarily in the lungs, and a nondetectable number of cells arrived in the tumor (119). These findings were consistent with previous work on the deformability of these cells using micropipet aspiration technique, in which interleukin-2 activation was shown to make these cells rigid, and their mechanical entrapment in the lung microcirculation was predicted (121, 145). Constitutive expression of certain adhesion molecules in the lung vasculature also facilitates their localization in the lungs (76).

One approach to reduce lung entrapment is to reduce the rigidity of these cells (122). Instead, to circumvent the lung, Melder et al injected A-NK cells into the blood supply of tumors and found that A-NK cells, both xenogenic and syngeneic, adhered to blood vessels in three different tumor models (120, 126, 146). These results also supported the hypothesis that the endogenous cells that adhere to tumor vessels after systemic interleukin-2 injection are mostly activated lymphocytes (138).

To find out which adhesion molecules are involved in the A-NK cell adhesion to tumor vessels, two in vitro approaches have been utilized. In the first approach, the tumor vasculature was simulated in vitro, by incubating the human umbilical vein endothelial cells in the tumor interstitial fluid collected using a micropore chamber (54, 80, 83, 124). Using targeted sampling fluorometry, Munn et al (128) were able to quantify the expression of relevant adhesion molecules on the human umbilical vein endothelial cell monolayers. To determine the relative contributions of these molecules in adhesion under physiological flow conditions, the flow chamber was utilized (129). By using appropriate antibodies, it was found that the molecules up-regulated on the human umbilical vein endothelial cells include intracellular adhesion molecule-1 and vascular cell adhesion molecule-1, which bind to CD18 and very late antigen-4 on the A-NK cells. Sporadic up-regulation of E-selectin was also observed, and the role of these molecules was confirmed in vivo by treating A-NK cells with antibodies against CD18 and very late antigen-4 prior to injecting them into the arterial supply of tumors. As in previous

in vitro studies, blocking these adhesion molecules nearly eliminated the adhesion of A-NK cells to tumor vessels (124).

What leads to the up-regulation of these molecules in the tumor vasculature? These molecules can be up-regulated by tumor necrosis factor alpha and a 90–kDa protein (p90) secreted by some neoplastic cells (85, 123, 125), and they can be down-regulated by transforming growth factor beta (45–47). To find out whether other molecules are present in the tumor milieu that also induce this up-regulation, and because tumor growth and metastasis are angiogenesis dependent, the two most potent angiogenic molecules—bFGF and VEGF/VPF—were studied (38, 39, 76). It was found that VEGF can mimic tumor interstitial fluid and up-regulate these molecules (30, 147). bFGF, on the other hand, exhibited no effect when used alone, but it abrogated the up-regulation induced by VEGF or tumor necrosis factor alpha (124). These findings were in concert with earlier reports that bFGF retards the transmigration of lymphocytes across endothelial monolayer (95) and reduces adhesion of endothelial cells to collagen at low cell density (62). They also offer a possible explanation for heterogeneous leukocyte–endothelial interactions in tumors; bFGF might have down-regulated adhesion molecules in these tumors. Current efforts are directed toward defining interactions between angiogenic and adhesion molecules using various in vitro and in vivo approaches, including genetically engineered mice (30, 76, 97, 161).

## PHARMACOKINETIC MODELING: SCALE UP FROM MOUSE TO HUMAN

Thus far, the steps in the delivery of molecules and cells to and within solid tumors have been analyzed. Can this information be integrated into a unified framework? The answer is yes, to some extent, using physiologically based pharmacokinetic modeling. This approach, pioneered by two chemical engineers in the 1960s, has been applied successfully to describe and scale up the biodistribution of low-molecular-weight agents (for reviews, see 26, 50, 70). This approach has been extended to macromolecules and cells (11, 12, 170–172).

In this approach, a mammalian body is represented by a number of physiological compartments interconnected anatomically. The volume and blood flow rate for each of these compartments/organs are known or can be measured. The parameters that characterize transport across the subcompartments (i.e. vascular, interstitial, and cellular) and the metabolism of various agents are not generally known and cannot be easily measured. One philosophy has been to use as many measured parameters as possible and to estimate the remaining parameters by fitting the model to the murine biodistribution data. By scaling up the parameters using well-defined scale-up laws (26), the biodistribution in human patients can be predicted and compared with clinical data. Discrepancies between predictions and actual data help in identifying interspecies differences and force the ques-

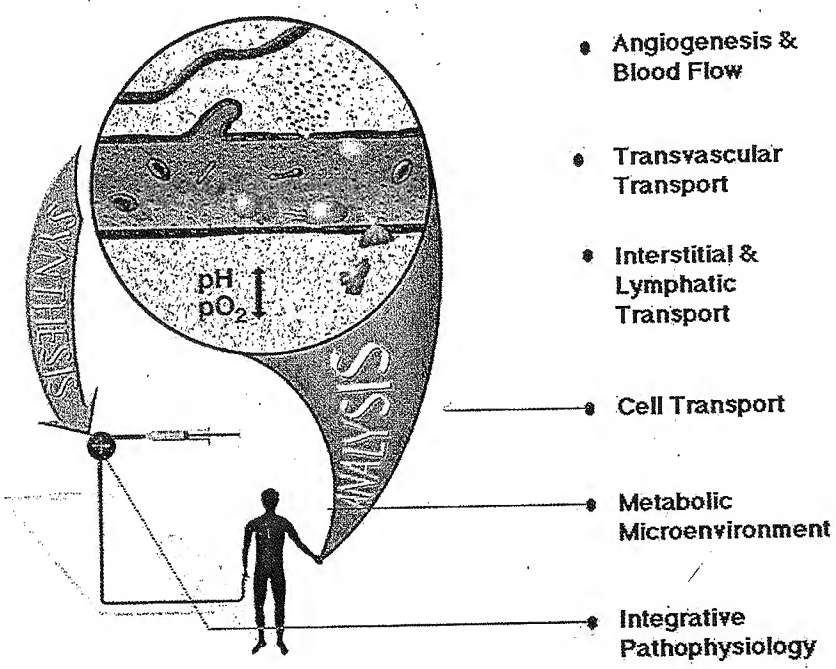


**Figure 1** Quantitative understanding of various steps involved in the delivery of therapeutic agents is studied by analyzing the underlying processes and then integrating the resulting information in a unified framework. More specifically, the goal of researchers is to develop a quantitative understanding of angiogenesis and blood flow, metabolic microenvironment, transvascular transport, interstitial and lymphatic transport, cell transport, and systemic distribution and interspecies scale-up.

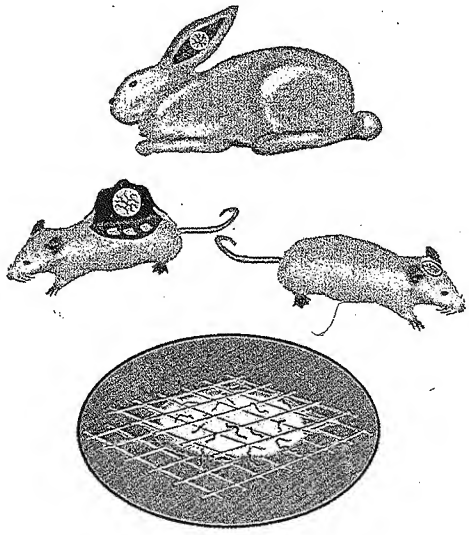

**Figure 2** Various microcirculatory preparations used to study delivery of therapeutic agents in solid tumors: (*top*) Sandison window in the rabbit ear (169); (*middle left*) Algire window in the dorsal skin of rodents (114); (*middle right*) cranial window in rodents (168); and (*bottom*) collagen I gel, containing angiogenic factors, sandwiched between nylon mesh (3 mm × 3 mm) to permit the growth of blood vessels (28). These preparations allow noninvasive, continuous measurement of angiogenesis and blood flow; metabolites, such as pH, $pO_2$; transport of molecules and particles; cell-cell interactions in vivo, and gene expression.

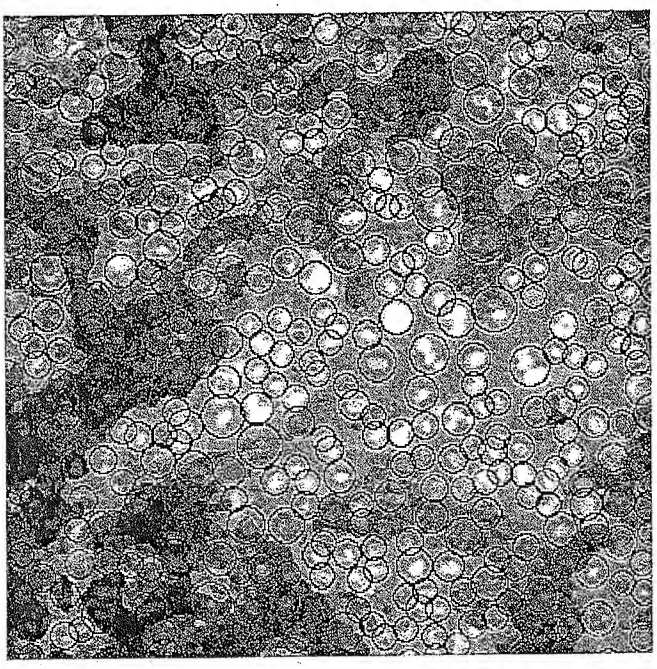

**Figure 3** Targeted sampling fluorometry provides quantification of adhesion molecule expression on the surface of endothelial cells in an intact monolayer. The *red* propidium iodide marks the cell nuclei, while the *green* antibody binds to adhesion molecules (VCAM-1 in this case). Using the cell nuclei as guides, the computer places appropriate regions of interest (*blue circles*) for measuring the green fluorescence of individual cells. (Adapted from Reference 128.)

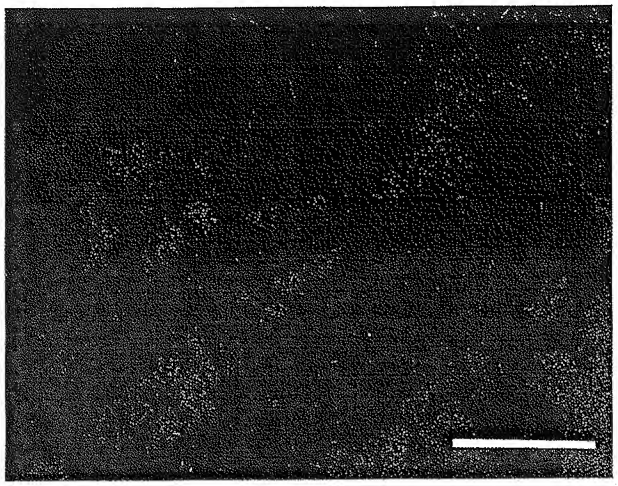

**Figure 7** Heterogeneous extravasation of 90–nm–diameter liposomes from LS174T tumor vessels, 48 h after injection. Note that some vessels are leaky, as indicated by the brighter fluorescence for rhodamine, whereas others are not. Extravasated liposomes do not diffuse far from blood vessels. (Adapted from Reference 167.)

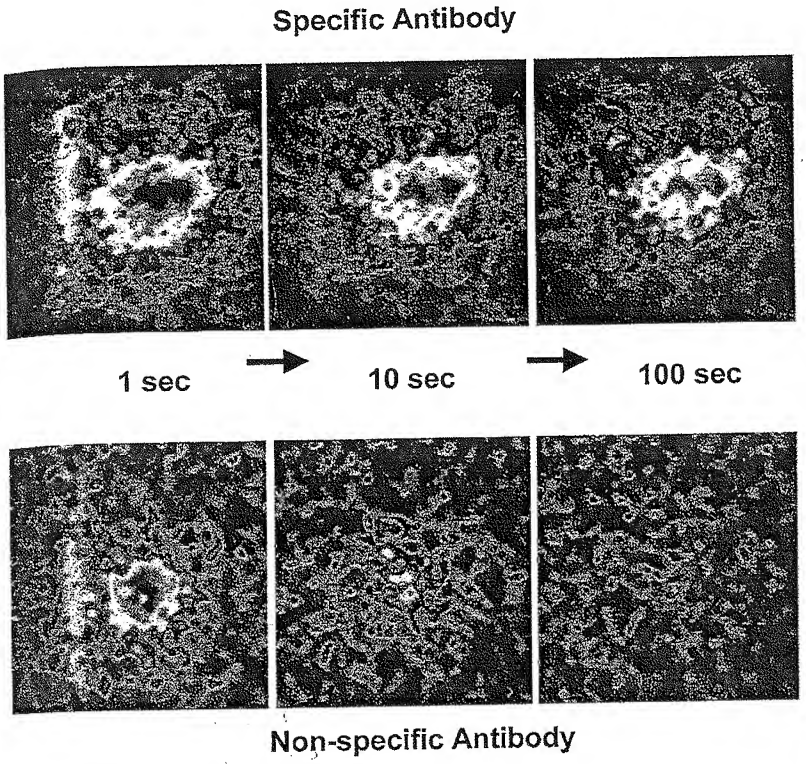


**Figure 8** Role of binding in the interstitial transport in tumors, measured using fluoresence recovery after photobleaching. (*Top*) Recovery is incomplete for an antibody against carcino-embryonic antigen, present on the surface of many carcinoma cells. (*Bottom*) Recovery of a photobleached spot is complete within approximately 100 s for a nonspecific monoclonal antibody. (Adapted from Reference 15.)

tioning of model assumptions. This is an evolutionary process—as understanding of underlying physiology and biochemistry improves, the relevant parameters are modified and the model is refined further. The model is useful not only for designing murine experiments and/or clinical trials, but also for identifying sensitive parameters that need careful measurement and analysis. If detailed spatial information about a tissue/organ is needed, then a distributed parameter model for that organ, e.g. tumor, must be developed (6–9, 11, 12, 63, 64, 82). Although simple in principle, this cyclic approach of analysis and synthesis has served as a useful paradigm for developing a deeper understanding of drug and cell distribution in normal and malignant tissues. The level of sophistication of these models is likely to improve as understanding of underlying principles grows (2).

## BENCH TO BEDSIDE

The physiologic factors that contribute to the heterogenous delivery of therapeutic agents to tumors include heterogeneous blood supply, interstitial hypertension, relatively long transport distances in the interstitium, and cellular heterogeneities (Figure 5). How can these physiologic barriers be exploited or overcome? Can findings about these barriers be taken from bench to bedside? Two recently developed strategies that have the potential to improve the detection and treatment of solid tumors in patients are described here.

As stated earlier, all solid tumors in patients exhibit interstitial hypertension (Table 1), provided the patient has not received any anti-edema treatment (22). Also, interstitial fluid pressure rises steeply in the tumor boundary (16, 74). This knowledge has been used to improve the design of the needle used by radiologists to localize the tumor for surgical excision (75). The needle placement in a tumor can be facilitated by placing a pressure-sensor in the needle. Because tumors begin to exhibit interstitial hypertension almost from the onset of angiogenesis (21), this needle may be able to help in localizing early disease. The same concept may be useful in optimizing location and infusion pressure of needles employed in intratumor infusion of therapeutic agents (17), and for monitoring response to therapy (176).

Several physical (e.g. radiation, heat) and chemical (e.g. vasoactive drugs) agents may lead to an increase in tumor blood flow or vascular permeability (32, 40, 43, 51, 53, 65, 81, 98, 99), or lower pH (80, 160). Another approach may be based on increasing the interstitial transport rate of molecules by increasing $K$ or $D$ enzymatically (17, 68, 155) or by using multistep approaches (9, 10, 12, 163). Several physical and chemical agents have been used to lower interstitial fluid pressure in tumors (53, 100, 103–105, 111, 112, 114, 177). Because microvascular and interstitial pressures in tumors are approximately equal, any change in one is followed rapidly by a similar change in the other, and thus the convective enhancement disappears rapidly (18, 133, 173, 174). By adapting a poroelastic model to solid tumors, it has been calculated theoretically and confirmed exper-

imentally that the time constant of pressure transmission across the tumor vasculature is on the order of 10 s (133). During such a short time period, the convective enhancement is calculated to be very small (~1%). However, if the vascular pressure is increased repeatedly and if the transvascular transport is unidirectional or if the molecule binds avidly in the extravascular region, then, in principle, drug delivery to solid tumors can be increased significantly (134).

In contrast, the physiologic barriers discussed here may be less of a problem for (*a*) radioimmunodetection, (*b*) treating leukemias, lymphomas, and small tumors (e.g. micrometastases) in which the physiological barriers are not yet fully established, (*c*) treatment of adequately perfused, low-pressure regions of large tumors for debulking, and (*d*) treatment with antibodies or other agents directed against the host cells (e.g. tumor endothelial cells, fibroblasts) or the subendothelial matrix. These physiologic barriers also may pose fewer problems for treatment with a molecule or cell that has nearly 100% specificity for cells in the tumor. Until such selective molecules or cells are developed, methods are urgently needed to overcome or exploit these physiologic barriers in tumors. It is hoped that an improved understanding of transport in tumors will help in developing these strategies (71–73).



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